STUDIES ON THE

Mg^{2+}-STIMULATED ADENOSINE TRIPHOSPHATASE

OF *ESCHERICHIA COLI* K-12

by

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A THESIS

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STATEMENT

All the experimental work reported in this thesis was performed by the author, unless specifically stated otherwise in the text.

David Fayle

(DAVID FAYLE)

PREFACE

This thesis describes the results of research carried out in the Department of Biochemistry, John Curtin School of Medical Research, Australian National University, Canberra, from April 1975 to May, 1978, under the general supervision of Dr. G. B. Cox and Professor F. Gibson.

Part of this work has been published or is in press:

"Characterization of the Mutant-*uncD*-Gene Product in a strain of *Escherichia coli K12*: an altered β-subunit of the magnesium ion-stimulated adenosine triphosphatase" FAYLE, D.R.H., DOWNIE, J.A., COX, G.B., GIBSON, F., and RADIK, J. (1978) *Biochemical Journal 172* (in press).

"Inhibition, by a Protease Inhibitor, of the solubilization of the F_1-portion of the Mg^{2+}-stimulated Adenosine Triphosphatase of *Escherichia coli*" COX, G.B., DOWNIE, J.A., FAYLE, D.R.H., GIBSON, F., AND RADIK, J. (1978) *Journal of Bacteriology 133*, 287-292.

(Abstract) "Altered β-subunits of the Mg-ATPase in *uncD* mutants of *E.coli*." -FAYLE, D.R.H., DOWNIE, J.A., COX, G.B., GIBSON, F., and RADIK, J. (1978) *Proceedings of the Australian Biochemical Society 11* (in press).

The abbreviations used without definition are those recommended by The Biochemical Journal. In addition, the following abbreviations were used:

Mg-ATPase or ATPase	:	Mg^{2+}-stimulated adenosine triphosphatase.
F_1-ATPase or F_1	:	The Mg-ATPase containing four or five types of subunit, as solubilized from the membrane.
F_0-sector	:	The membrane-sector of the Mg-ATPase, left when the F_1-ATPase is removed.
F_1-F_0 ATPase or F_1-F_0	:	The membrane Mg-ATPase complex, *in situ* or as solubilized by detergent.
TF_1-F_0 ATPase, TF_1-F_0, TF_1 or TF_0	:	Mg-ATPase complexes from the thermophilic bacterium PS3.
bisacrylamide	:	N, N'-methylene bisacrylamide.
C(%)	:	the percentage by weight of bisacrylamide/(acrylamide + bisacrylamide).
CCCP	:	carbonyl cyanide *m*-chlorophenylhydrazone.
DCCD	:	N, N'-dicyclohexylcarbodiimide.
Δp	:	protonic (proton-motive) potential difference, or proton-motive force.
ΔpH	:	pH gradient across the membrane.
$\Delta \psi$	:	membrane electrical potential.
EACA	:	ε-amino-n-hexanoic acid (or ε-amino-n-caproic acid).
H^+/P	:	the ratio of protons translocated to ATP hydrolysed or P_i esterified.
Hepes	:	4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid.
M.W.	:	molecular weight.
NP-40	:	Nonidet P-40; octylphenoxy-polyethoxy-ethanol, containing 7-9 mol/mol of ethoxy linkages (Triton X-100 is similar, but contains 9-10

mol/mol of ethoxy linkages).

PAB	:	*p*-aminobenzamidine.
p.f.u.	:	plaque-forming units.
SDS	:	sodium dodecyl sulphate.
STEM	:	a buffer containing sucrose, Tes, EGTA, Mg-acetate and EACA (Chapter II,G).
T(%)	:	the percentage by weight of acrylamide + bisacrylamide.
Temed	:	N, N, N', N'-tetramethylethylenediamine.
Tes	:	2-[(2-hydroxy-1, -1-bis (hydroxymethyl) ethyl) amino] ethanesulphonic acid.

The thesis is divided into ten chapters. The first chapter is a general introduction, including references to literature up to April, 1978. The materials and methods are described in Chapter II, followed by seven chapters describing experimental work, which include brief introductory sections and comprehensive discussions. The final chapter is a short general discussion of the results, placed in a wider context.

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ABSTRACT

Mutant strains of *Escherichia coli* K-12 lacking both Mg^{2+}-stimulated adenosine triphosphatase activity and oxidative phosphorylation (*unc* mutants) had previously been isolated. Two such mutant strains, carrying point mutations, in the *uncD* gene, were found to have detectable alterations in the β-subunit of the adenosine triphosphatase, and the conclusion was drawn that the *uncD* gene is the structural gene for the β-subunit.

A novel selective method is described for the solubilization, from the cytoplasmic membrane, of an adenosine triphosphatase complex containing five types of subunit. The method is based on the prevention, by the protease inhibitor *p*-aminobenzamidine, of the solubilization of the enzyme during washing of the membranes at low-ionic strength. When *p*-aminobenzamidine was subsequently removed, the enzyme was solubilized.

A new procedure is described for the two-step purification of the solubilized adenosine triphosphatase complex. The method is two-dimensional gel electrophoresis, using isoelectric focusing in the first dimension, followed by pore gradient electrophoresis in the second dimension.

The two-dimensional analytical gel electrophoresis procedure of O'Farrell (1975), in which proteins are dissociated into their constituent polypeptide chains, was modified to improve the resolution of membrane polypeptides. Using a combination of the semi-preparative and analytical electrophoresis techniques, a comparison was made of

the adenosine triphosphatase complexes solubilized either by the selective method already mentioned, or by the extraction of the other membrane components into chloroform. The former complex contained all five types of subunit (α, β, γ, δ, ε) whilst the latter complex lacked the δ-subunit. The semi-preparative electrophoresis technique was additionally found to be useful for the partial resolution of sub-species of these adenosine triphosphatase complexes.

The analytical electrophoresis procedure was used for the detection of abnormal β-subunits in mutant strains lacking adenosine triphosphatase activity, carrying either the *uncD409* or *unc-405* alleles. The latter mutant was thus confirmed as an *uncD* mutant. The behaviour of the abnormal β-subunits and of the α-subunit , was monitored during the fractionation procedures normally used for the solubilization of the adenosine triphosphatase from the membrane. The abnormal β-subunits of both the *uncD409* and the *uncD405* mutants were refractory towards these procedures. These observations were correlated with other data on the abnormal proton-permeability of various membrane fractions from both strains, and also with data on the ability or inability of such membranes to bind added normal adenosine triphosphatase to reconstitute ATP-driven activities.

Using a 'localized' mutagenesis technique, two further *unc* mutant strains were isolated. One of these strains (AN1007, *unc-436*) was characterized by genetic complementation tests, membrane polypeptide analysis, and measurement of energy-linked activities. The mutation was found to be a polar point mutation in the *unc* operon, affecting the *uncD* and *uncC* genes. A normal α-subunit of the adenosine triphosphatase was present, but the β-subunit was either absent or altered

substantially. Despite the lack of the adenosine triphosphatase complex, however, the membranes retained a low permeability to protons. Moreover, the addition of normal adenosine triphosphatase to membranes from the mutant strain did not reconstitute ATP-dependent activities. This mutant is compared with other mutant strains in a wider context in the general discussion.

Lastly, the polypeptides present in the membrane adenosine triphosphatase complex from the inner membrane of yeast mitochondria, were compared with those of the solubilized enzyme from *E.coli*. The α-, β-, γ- and δ-subunits were found to be very similar, but not identical, in both organisms. The evolutionary significance of this observation is discussed.

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Chapter I.

INTRODUCTION

" ..... here we have found a machine which has facilitated the bulk of energy transduction on this planet. It is highly efficient at ambient temperature, non-polluting, non-explosive and we know that it works. We may be able to apply the principles, when we understand them, to our own devices."

A. E. Senior (1978).

The adenosine triphosphatase (ATPase; EC 3.6.1.3) catalyses the synthesis and breakdown of ATP in organisms as diverse as mammals, plants, fungi, and strictly anaerobic bacteria (Senior, 1973, 1978; Haddock and Jones, 1977; Konings and Boonstra, 1977; Harold, 1977a). The ATPase is ubiquitous in membranes containing electron-transfer systems, and is able to conserve energy, released during electron transport, by synthesizing ATP. ATP produced in this way, or by substrate-level phosphorylation, can also be hydrolysed by the membrane ATPase (hence the term "ATPase") in such a way that the energy released can be used to drive energy-requiring reactions associated with the membrane. It has become apparent in recent years that the structure and general properties of the membrane ATPase have been conserved to a remarkable extent during evolution, and this conservation is emphasized further by the results of a comparison made between the ATPases of the bacterium *Escherichia coli* and the yeast *Saccharomyces cerevisiae*, described in Chapter IX of this thesis. In bacterial cell membranes, mitochondrial inner membranes, chloroplast thylakoid membranes, or the chromatophores of photosynthetic bacteria, the ATPase consists of

a large protein, containing at least two hydrophobic 'membrane-sector' ('F_0') polypeptides, four to five types of 'F_1-sector' polypeptides which can be isolated together as an 'F_1-ATPase' protein in aqueous solution, and a variable number of peripheral polypeptides which are found in the purified F_1-F_0 ATPase complex, isolated in the presence of detergent (Senior, 1973, 1978; Haddock and Jones, 1977; Sebald, 1977; Abrams and Smith, 1974). There is some evidence (see Senior, 1973) that the F_1-sector protrudes from the membranes as a 'knob', perhaps attached to the F_0-sector by 'stalk' polypeptides. Since Mg^{2+} ions are required for ATPase activity, the ATPase has been termed the Mg^{2+}-stimulated ATPase (Mg-ATPase), and this terminology is used for the enzyme from *E.coli* throughout this thesis. The designation 'ATP synthase (or synthetase)' is not appropriate for the detergent-solubilized F_1-F_0 ATPase or the solubilized Mg-ATPase, since ATP synthetic activity has not been demonstrated in such preparations.

Despite intensive efforts over the last 25 years, the mechanisms of energy-linked ATP synthesis and hydrolysis catalyzed by the Mg-ATPase are still unclear (Boyer, Chance, Ernster, Mitchell, Racker and Slater, 1977; Williams, 1978; Harris, 1978). Moreover, very little is known about the structural and functional role of each subunit of the Mg-ATPase complex, since the intricacies of the Mg-ATPase protein and its membrane environment have impeded such investigations. A related area of current interest is the biosynthesis of the Mg-ATPase, both in the mitochondria of moulds and yeasts (Sebald, 1977; Linnane *et al*, 1976; Senior, 1978), and in *E.coli* (Gibson *et al*, 1978).

The work described in this thesis was undertaken with the objective of identifying the polypeptides affected in various mutant

strains of *E.coli* containing defective Mg-ATPase. The aim of this work was to provide a basis for the eventual definition of the roles of the various subunits, and the mechanisms of ATP synthesis and hydrolysis, as well as the elucidation of the biosynthesis of the Mg-ATPase.

A. THE USE OF BIOCHEMICAL GENETICS IN THE STUDY OF ENERGY-LINKED REACTIONS

The use of biochemical genetics to elucidate metabolic processes, and in particular, biosynthetic pathways, has led to a very rapid expansion during the last 30 years in the understanding of these processes. As observed in 1973 by Gibson and Cox, the genetic approach had not been extensively used in the field of oxidative phosphorylation, and progress had been correspondingly slow. More recently, the value of biochemical genetics in bioenergetic research has been widely-recognized, and already considerable progress has been made towards the elucidation of the structural and functional roles of the Mg-ATPase (Cox and Gibson, 1974; Simoni and Postma, 1975; Haddock and Jones, 1977; Sebald, 1977). Substantial progress has been made in locating the genes involved in the biosynthesis of the Mg-ATPase. In yeast, the structural genes for the F_1-subunits are in nuclear DNA, whilst those for the F_0-subunits are in mitochondrial DNA (See Senior, 1978; Sebald, 1977). Tzagoloff *et al* (1976) and Marahiel *et al* (1977) have identified mitochondrial mutations which affect the gene coding for an F_0-sector protein of 6-8000 daltons. Linnane *et al* (1976) have indirect evidence that a second mitochondrial mutation affects an F_0-sector protein of 20,000 daltons.

The bacterium *E.coli* K12 has proved particularly useful in such studies. This organism is non-pathogenic, grows rapidly in defined synthetic media, and has an extensively-characterized chromosome, on which over 650 genes have been mapped (Bachmann *et al*, 1976). Moreover, the genotype can be easily manipulated, and strains of the required type isolated, using a variety of well-established and powerful techniques (Hayes, 1968). It was for these reasons that *E.coli* was selected for studies into oxidative phosphorylation (Butlin *et al*, 1971; Gibson and Cox, 1973), and more recently for studies into the interrelated aspects of the synthesis and assembly of the subunits of the Mg-ATPase protein, the structural and functional roles of the subunits, and the mechanisms of ATP synthesis and hydrolysis.

B. UNCOUPLED (*UNC*) MUTANT STRAINS OF *ESCHERICHIA COLI*, AND THE ROLE OF THE *UNC* GENE PRODUCTS IN ENERGY TRANSDUCTION

(a) Uncoupled (*unc*) strains selected by inability to grow on non-fermentable carbon sources

Mutant strains of *E.coli* unable to grow on non-fermentable substrates such as succinate, malate, or lactate, can be obtained after mutagenesis and selection on appropriate media. Of the strains obtained in this way, some were found to be defective in the 'coupling' of electron-transport to ATP synthesis, and in the energization by ATP of certain reactions associated with the membrane, and these mutants were therefore designated *unc* (for 'uncoupled') (Butlin *et al*, 1971, 1973; Cox and Gibson, 1974). As indicated above, both synthesis and

hydrolysis of ATP are catalyzed by the membrane ATPase, and *unc* mutants are defective in these functions (Haddock and Jones, 1977). The *unc* mutants so far characterized are listed in Tables I.1, 2 and 3. Most of these strains are derived from strain K-12.

Some workers have screened mutant strains resistant to the antibiotic neomycin, and found *unc* mutants amongst these strains (Tables I.1 and 2; Kanner and Gutnick, 1972a; Rosen, 1973; Yamamoto *et al*, 1973; Kanner *et al*, 1975). These *unc* mutants had similar characteristics to those selected solely by lack of growth on succinate, malate or lactate.

(b) Characteristics of *unc* mutant strains

The common characteristics of the *unc* strains are as follows (Gibson and Cox, 1973; Cox and Gibson, 1974):

(i) they cannot grow on non-fermentable carbon sources;

(ii) aerobic growth yields on limiting glucose are mid-way between the aerobic and anaerobic growth yields of a normal strain;

(iii) respiration is normally unimpaired;

(iv) oxidative phosphorylation is not detectable;

(v) in membrane preparations, the ATP-driven pyridine nucleotide transhydrogenase activity is absent; respiration-driven transhydrogenase is present, but in some strains is secondarily affected;

(vi) in membrane preparations, 'energization' of the membrane by ATP is defective, whilst respiration-

driven energization is either present or secondarily affected. The energization of membranes can be monitored by the quenching of acridine dye fluorescence (Nieuwenhuis *et al*, 1973; Haddock and Downie, 1974) or by energy-linked reactions such as the transhydrogenase (point (v)).

(vii) all *unc* mutations so far described are located in a region of the *E.coli* chromosome (min 83) which is co-transducible with the *ilv* locus (Bachmann *et al*, 1976; Haddock and Jones, 1977). Recently it has been shown that the *unc* genes are arranged in an operon (Gibson *et al*, 1978).

The *unc* mutant strains can generate sufficient ATP through glycolysis to support growth, and can thus grow on glucose aerobically. In normal strains, the oxidation of lactate, or of intermediates of the tricarboxylic acid cycle, is coupled to phosphorylation of ADP catalysed by the Mg-ATPase. Since the *unc* mutant strains are defective in this enzyme, they cannot use the energy derived from oxidation of such compounds to generate ATP, and so cannot grow at the expense of these compounds.

In general, *unc* mutants can be classified either as having Mg-ATPase activity ($ATPase^+$; Table I.2) or lacking it ($ATPase^-$; Table I.1). Although both classes of *unc* mutants have similar aerobic growth characteristics, mutants of the former class ($ATPase^+$) carrying the *uncB402* or *uncC424* alleles could grow anaerobically on glucose without added electron acceptors, whereas $ATPase^-$ strains could not (Butlin *et al*, 1973; Cox *et al*, 1974; Gibson *et al*, 1977b). However,

TABLE I.1

SOME PROPERTIES OF MUTANT STRAINS OF *ESCHERICHIA COLI* LACKING MG-ATPASE ACTIVITY. ACTIVITIES WERE MEASURED IN MEMBRANE PREPARATIONS EXCEPT WHERE INDICATED. WHERE THEY HAVE BEEN MEASURED, OXIDATIVE PHOSPHORYLATION, ATP-DRIVEN TRANSHYDROGENASE ACTIVITY, ATP-DEPENDENT QUENCHING OF ACRIDINE-DYE FLUORESCENCE, AND ATP-DEPENDENT ACTIVE TRANSPORT WERE ABSENT IN UNWASHED MEMBRANES. 'STRIPPED' MEMBRANES HAVE BEEN SUBJECTED TO PROCEDURES WHICH REMOVE THE MG-ATPASE ACTIVITY FROM MEMBRANES OF A NORMAL STRAIN. IN THE RECONSTITUTION EXPERIMENTS, THE UNBRACKETED RESULTS REFER TO RECONSTITUTION WITH F_1-ATPASE, WHEREAS THOSE IN SQUARE BRACKETS REFER TO RECONSTITUTION WITH DCCD.

CHARACTERISTIC	STRAIN: 7 *UNCA401*	32 *UNC-103C*	33 DG20/1 BH212	11 *UNC-405*	33 AS12/25	13 MDA_1	13 MDA_2	12 *UNCD409*	33 BH273 AS69/1	13 MDA_3	19 N_{I44}	27 NR70	29 NR76	40 e.g. *UNC-17*	35 DL-54
SUBUNITS OF MG-ATPASE AND TYPE OF INACTIVE COMPLEX	'NORMAL' COMPLEX 11 23 α β γ δ ε 6			SEE CHAPTER VII				SEE CHAPTER VI			No α, β or γ 21	No α, β or γ 4	SMALLER COMPLEX 1		α AND/OR β 4
NORMAL MEMBRANE SECTOR (F_0)	+ 9 10		+	SEE CHAPTER VII		+	+	SEE CHAPTER VI				+	+		+ 5
UNWASHED MEMBRANES — RESPIRATION-DRIVEN ACTIVITIES	+ 10 14		+	SEE TEXT	+			+	+		SEE TEXT	LOW 37	LOW 29		LOW 5
UNWASHED MEMBRANES — ACTIVE TRANSPORT	+ 26 18 25										SEE TEXT	LOW 27 28 4 17	LOW 29		LOW 2 4
RECONSTITUTION OF UNWASHED MEMBRANES WITH ACTIVE MG-ATPASE OR (DCCD) — BINDING OF MG-ATPASE						PARTIAL	+			−					
RECONSTITUTION OF UNWASHED MEMBRANES WITH ACTIVE MG-ATPASE OR (DCCD) — OXIDATIVE PHOSPHORYLATION			PARTIAL		+				−						
RECONSTITUTION OF UNWASHED MEMBRANES WITH ACTIVE MG-ATPASE OR (DCCD) — ATP-DRIVEN ACTIVITIES	− 11		PARTIAL	PARTIAL 11 15	+				−				+ 29		+ 5
RECONSTITUTION OF UNWASHED MEMBRANES WITH ACTIVE MG-ATPASE OR (DCCD) — RESPIRATION-DRIVEN ACTIVITIES			+	SEE CHAPT. VII	+						[+] 25	+[+] 38	+[+] 29		+[+] 5
RECONSTITUTION OF UNWASHED MEMBRANES WITH ACTIVE MG-ATPASE OR (DCCD) — ACTIVE TRANSPORT											[+] 39	[+] 27 28 36 4	[+] 29		+ [+] 2
STRIPPED MEMBRANES — RESPIRATION-DRIVEN ACTIVITIES	− 18 SEE TEXT		−	SEE CHAPT. VII	+			+ SEE CHAPT. VI	+		LOW 25	LOW 17	LOW 28		LOW 5
STRIPPED MEMBRANES — ACTIVE TRANSPORT	− 18											LOW 17			
RECONSTITUTION OF STRIPPED MEMBRANES WITH ACTIVE MG-ATPASE OR (DCCD) — BINDING OF MG-ATPASE						+	+			−	LOW 25				
RECONSTITUTION OF STRIPPED MEMBRANES WITH ACTIVE MG-ATPASE OR (DCCD) — OXIDATIVE PHOSPHORYLATION	+ 10		+		PARTIAL				−						
RECONSTITUTION OF STRIPPED MEMBRANES WITH ACTIVE MG-ATPASE OR (DCCD) — ATP-DRIVEN ACTIVITIES	+ 9 10		+	+ 11	PARTIAL	+	+	− SEE CHAPT. VI	−	−	− 5	+ 37 17	+ 29		+ 5
RECONSTITUTION OF STRIPPED MEMBRANES WITH ACTIVE MG-ATPASE OR (DCCD) — RESPIRATION-DRIVEN ACTIVITIES	+ [+] 18		+	SEE CHAPT. VII + (+)							− [+] 5 25	+[+] 37 17	+[+] 29		+ [+] 5
RECONSTITUTION OF STRIPPED MEMBRANES WITH ACTIVE MG-ATPASE OR (DCCD) — ACTIVE TRANSPORT	[+] 18											+[+] 36			+ 34
ACTIVE TRANSPORT IN WHOLE CELLS — CELLS GROWN $+O_2$; UPTAKE $+O_2$		+ 32	+	+ 30					+		+ 39			LOW 40	VARIABLE 34 3
ACTIVE TRANSPORT IN WHOLE CELLS — UPTAKE $+O_2$ (+DCCD)														[+] 40	
ACTIVE TRANSPORT IN WHOLE CELLS — $-O_2$ + FUMARATE OR NITRATE; UPTAKE $-O_2$ + FUMARATE OR NITRATE				+ 30										+ 40	
OXIDASE ACTIVITIES	+ 7 26	+ 31 32				+	+			+	+ 16	+ 23			+ 35
UNCOUPLED GROWTH YIELD	+ 7			+ 11		+	+	+		+	+	+		+ 40	+ 35
ANAEROBIC GROWTH ON GLUCOSE ONLY	− 8		−	− 11	−	−	−		−	−					

REFERENCES

1. Adler & Rosen, 1976
2. Altendorf *et al*, 1974
3. Berger, 1973
4. Boonstra *et al*, 1975
5. Bragg & Hou, 1973
6. Bragg & Hou, 1977
7. Butlin *et al*, 1971
8. Butlin *et al*, 1973
9. Cox *et al*, 1973a
10. Cox *et al*, 1973b
11. Cox *et al*, 1974
12. Cox *et al*, 1978a
13. Daniel *et al*, 1975
14. Gibson *et al*, 1977a
15. Gibson *et al*, 1977b
16. Gutnick *et al*, 1972
17. Hasan & Rosen, 1977
18. Hasan *et al*, 1978
19. Kanner & Gutnick, 1972a
20. Kanner & Gutnick, 1972b
21. Kanner *et al*, 1975
22. Kobayashi *et al*, 1974
23. Maeda *et al*, 1976
24. Nieuwenhuis *et al*, 1973
25. Patel & Kaback, 1976
26. Prezioso *et al*, 1973
27. Rosen, 1973a
28. Rosen, 1973b
29. Rosen & Adler, 1975
30. Rosenberg *et al*, 1975
31. Schairer & Gruber, 1973
32. Schairer & Haddock, 1972
33. Schairer *et al*, 1976
34. Simoni & Postma, 1975
35. Simoni & Shallenberger, 1972
36. Tsuchiya & Rosen, 1975a
37. Tsuchiya & Rosen, 1975b
38. Tsuchiya & Rosen, 1977
39. Van Thienen & Postma, 1973
40. Yamamoto *et al*, 1973

TABLE I.2

SOME PROPERTIES OF MUTANT STRAINS OF *ESCHERICHIA COLI* AFFECTED IN *UNC* GENES, BUT WHICH RETAIN Mg-ATPase ACTIVITY. STRAINS RF-7, DC1, *ETC-15* AND *UNC-373* CANNOT BE CONSIDERED AS UNCOUPLED STRAINS IN THE NORMAL SENSE (SEE TEXT); ALL OTHER STRAINS LACK OXIDATIVE PHOSPHORYLATION, ATP-DRIVEN TRANSHYDROGENASE ACTIVITY, ATP-DEPENDENT QUENCHING OF ACRIDINE DYE FLUORESCENCE, AND ATP-DEPENDENT ACTIVE TRANSPORT, WHERE THESE ACTIVITIES HAVE BEEN MEASURED. 'STRIPPED' MEMBRANES HAVE BEEN SUBJECTED TO PROCEDURES WHICH REMOVE Mg-ATPase FROM MEMBRANES OF A NORMAL STRAIN. IN THE RECONSTITUTION EXPERIMENTS, THE UNBRACKETED RESULTS REFER TO RECONSTITUTION WITH F_1-ATPase, WHEREAS THOSE IN SQUARE BRACKETS REFER TO RECONSTITUTION WITH DCCD.

CHARACTERISTIC \ STRAIN	3 *UNCB402*	22 DG 15/10 DG 26/4	22 DG 7/10 DG 31/3	21 *UNC-253*	20 BG-31	7 MDB	15 B_{V4}	11 6 *UNCC424*	17 NEOMYCIN-RESISTANT K_{I1} A_{I44}	23 *UNC-373*	14 *ETC-15*	8 RF-7 *(DCC-1)*	9 DC_1
OXIDATIVE PHOSPHORYLATION	— 3 24	—	—	—			— 12	— 11		—		+ 8	+
NORMAL MG-ATPASE (F_1)	+ 5 16 2	+	+ (LABILE)				+ 15 α, β and γ	SEE TEXT	+ 15 α, β and γ	— SEE TEXT	LOW SPECIFIC ACTIVITY ALTERED γ	+ 8	+
MG-ATPASE DCCD-SENSITIVE	— 8 6 13	—	—		—	—	— 12	SEE TEXT	— 17			— 8	—
MG-ATPASE LOOSELY-BOUND TO MEMBRANE AND/OR PRESENT IN CYTOPLASM	+ 6 11				+		+ 12 17 15	+ 11	+ 15				
MG-ATPASE FROM MUTANT STRAIN RECONSTITUTES NORMAL STRIPPED MEMBRANES	+ 4	+	+			—	— 15	SEE TEXT	+ 15		+ 1	+ 8	+
NORMAL MEMBRANE SECTOR (F_0)	—	—	—		—				— 17			— 8	—
UNWASHED MEMBRANES — RESPIRATION-DRIVEN ACTIVITIES	+ 4 10 13	+	+		+		LOW 17	+ 11	LOW 17		— 1		
UNWASHED MEMBRANES — ACTIVE TRANSPORT	+ 13								— 25		— 14	+ 18	
UNWASHED MEMBRANES — ATP-$^{32}P_i$ EXCHANGE		—	—						15				
RECONSTITUTION OF UNWASHED MEMBRANES WITH ACTIVE MG-ATPASE OR (DCCD) — BINDING OF MG-ATPASE						—							
RECONSTITUTION OF UNWASHED MEMBRANES WITH ACTIVE MG-ATPASE OR (DCCD) — OXIDATIVE PHOSPHORYLATION									— 15				
RECONSTITUTION OF UNWASHED MEMBRANES WITH ACTIVE MG-ATPASE OR (DCCD) — ATP-DRIVEN ACTIVITIES							[−] 17		− [+] 15 17 25 (PARTIAL)				
RECONSTITUTION OF UNWASHED MEMBRANES WITH ACTIVE MG-ATPASE OR (DCCD) — RESPIRATION-DRIVEN ACTIVITIES													
RECONSTITUTION OF UNWASHED MEMBRANES WITH ACTIVE MG-ATPASE OR (DCCD) — ACTIVE TRANSPORT													
STRIPPED MEMBRANES — RESPIRATION-DRIVEN ACTIVITIES	+ 4 13	+	+		+		LOW 17		— 17				
STRIPPED MEMBRANES — ACTIVE TRANSPORT	+ 13											— 18	
RECONSTITUTION OF STRIPPED MEMBRANES WITH ACTIVE MG-ATPASE OR (DCCD) — BINDING OF MG-ATPASE						—	— 17		— 17		LOW 1		+
RECONSTITUTION OF STRIPPED MEMBRANES WITH ACTIVE MG-ATPASE OR (DCCD) — OXIDATIVE PHOSPHORYLATION	— 4	—	—										
RECONSTITUTION OF STRIPPED MEMBRANES WITH ACTIVE MG-ATPASE OR (DCCD) — ATP-DRIVEN ACTIVITIES	— 4	—	—			—	— 17		— 17 15		LOW 1		
RECONSTITUTION OF STRIPPED MEMBRANES WITH ACTIVE MG-ATPASE OR (DCCD) — RESPIRATION-DRIVEN ACTIVITIES							−[−] 17		− [+] 17 15				
RECONSTITUTION OF STRIPPED MEMBRANES WITH ACTIVE MG-ATPASE OR (DCCD) — ACTIVE TRANSPORT	[−] 13											[−] 18	
ACTIVE TRANSPORT IN WHOLE CELLS — CELLS GROWN $+O_2$, UPTAKE $+O_2$	+ 19	+	+	+	+				+ 25				
ACTIVE TRANSPORT IN WHOLE CELLS — CELLS GROWN $-O_2$, UPTAKE $-O_2$													
ACTIVE TRANSPORT IN WHOLE CELLS — CELLS GROWN +FUMARATE OR NO_3^-, UPTAKE +FUMARATE OR NO_3^-	+ 19												
OXIDASE ACTIVITIES	+ 3			+		+	+ 12 15	+ 11	+ 17	+	+ 14		
UNCOUPLED GROWTH YIELD	+ 3 10					+		+ 11		+			
ANAEROBIC GROWTH ON GLUCOSE ONLY	+ 3 19	—	—			—		+ 11		+			

1. Bragg *et al*, 1973.
2. Bragg and Hou, 1977.
3. Butlin *et al*, 1973.
4. Cox *et al*, 1973b.
5. Cox *et al*, 1974.
6. Cox *et al*, 1977.
7. Daniel *et al*, 1975.
8. Fillingame, 1975.
9. Friedl *et al*, 1977.
10. Gibson *et al*, 1977a.
11. Gibson *et al*, 1977b.
12. Gutnick *et al*, 1972.
13. Hasan *et al*, 1978.
14. Hong and Kaback, 1972.
15. Kanner *et al*, 1975.
16. Maeda *et al*, 1976.
17. Nieuwenhuis *et al*, 1973.
18. Patel and Kaback, 1976.
19. Rosenberg *et al*, 1975.
20. Simoni & Shandell, 1975.
21. Schairer and Gruber, 1973.
22. Schairer *et al*, 1976.
23. Thipayathasana, 1975.
24. Tsuchiya, 1977.
25. Van Thienen and Postma, 1973.

ATPase$^+$ *unc* mutants isolated by Daniel *et al* (1975) and Schairer *et al* (1976) could not grow on glucose anaerobically (Table I.2). The ability to grow under these conditions is related to the ability of the strain to generate sufficient fumarate, both to induce the fumarate reductase, and also to support energy-requiring activities by electron-transport to fumarate via the fumarate reductase system (Rosenberg *et al*, 1975; Konings and Boonstra, 1977).

(c) Membrane-associated activities linked to the hydrolysis of ATP catalyzed by the Mg-ATPase

Energy released by the hydrolysis of ATP, catalyzed by the Mg-ATPase, can be used to drive a number of energy-requiring processes associated with the cytoplasmic membrane. It is now well-established that the pyridine nucleotide transhydrogenase, which catalyzes the reduction of NADP$^+$ by NADH, can be driven by ATP, or alternatively by electron transport (see Cox and Gibson, 1974). The reverse reaction, i.e. the reduction of NAD$^+$ by NADPH, does not require energy (Cox *et al*, 1971; Cox and Gibson, 1974). It is also clear that the active transport or facilitated diffusion of many ions and metabolites requires an 'energized' state of the membrane, derived from ATP hydrolysis or from electron-transport (Simoni and Postma, 1975). The most clear-cut evidence for the involvement of the Mg-ATPase in the provision of energy for the translocation of metabolites and ions has in fact come from studies with *unc* mutants (Schairer and Haddock, 1972; see Simoni and Postma, 1975; Tsuchiya and Rosen, 1975b). The involvement of ATP hydrolysis, via the Mg-ATPase, in providing the energy required for motility and chemotaxis in *E.coli* has also been investigated (Larsen *et al*, 1974; Thipayathasana and Valentine, 1974;

Koshland, 1977).

(d) Oxidative phosphorylation

Studies carried out by Butlin *et al* (1971) using the first *unc* mutant isolated (carrying the *uncA401* allele, Table I.1) provided compelling evidence for the involvement of the Mg-ATPase of *E.coli* in oxidative phosphorylation, as measured in everted (Haddock and Jones, 1977) membrane vesicles and also deduced from the growth characteristics of the strain. Oxidative phosphorylation has also been measured in whole cells (Hempfling, 1970) and is lacking in *unc* mutant strains (Gutnick *et al*, 1972; Schairer and Gruber, 1973), but this approach is open to a number of criticisms (Jones, 1977). Intensive studies on the growth yields obtained per mole of molecules such as succinate or glucose, have confirmed the relationship of such growth yields to the efficiency of oxidative phosphorylation (Stouthamer, 1977; Jones, 1977; Jones *et al*, 1977). Finally, oxidative phosphorylation in response to an artifically-imposed electrochemical gradient has been measured, but is lacking in *unc* mutants, in both vesicles (Tsuchiya and Rosen, 1976; Tsuchiya, 1977) and whole cells (Wilson *et al*, 1976).

(e) The structural role of the Mg-ATPase in the proton-permeability and respiration-linked functions of the cytoplasmic membrane

There is now considerable evidence for a specific involvement of the membrane Mg-ATPase structure in the proton conductance of the cytoplasmic membrane (Haddock and Jones, 1977; Okamoto *et al*, 1977).

The native membrane, containing intact F_1-F_0 ATPase complexes, normally has a low proton conductance (Rosen, 1973b). The membranes can be stripped of F_1 subunits by low-ionic strength washing (Bragg and Hou, 1972), or by treatment with chaotropic agents such as urea or guanidine-HCl (Bragg and Hou, 1977; Hasan and Rosen, 1977). The rate of proton equilibration across the resulting 'stripped membrane' is higher than that across the native membrane (Patel *et al*, 1975; Patel and Kaback, 1976; Hasan and Rosen, 1977). When an intact solubilized Mg-ATPase (F_1) is added back to such stripped membranes, the rate of proton equilibration is reduced, such that the reconstituted membrane resembles the native membrane rather than the stripped membrane (Hasan and Rosen, 1977). In short, membranes are more 'leaky' to protons when the Mg-ATPase (F_1) is absent than when it is present, and the F_0 sector forms a channel allowing rapid passive translocation of protons. The F_0-sector of the Mg-ATPase from the thermophilic bacterium PS3 has recently been purified and shown to be a passive proton conductor, inhibited by DCCD, F_1, or antibody against F_0, when reconstituted into vesicles (Okamoto *et al*, 1977). The term 'F_0' as used here includes only those proteins essential for the terminal step in phosphorylation, energized for instance by an artificially imposed electrochemical proton gradient across a vesicle containing purified F_1-F_0 Mg-ATPase (Sone *et al*, 1977a, b).

In three ATPase$^-$ mutant strains, the membranes have been shown to have an elevated permeability to protons. These strains, which are listed in Table I.1, are strains NR70 (Rosen, 1973b), DL-54 (Altendorf *et al*, 1974) and NR76 (Rosen and Adler, 1975). Strain NR70 lacks any cross-reacting material to antibodies against the larger subunits (α, β or γ) of the Mg-ATPase (Boonstra *et al*, 1975). Strain DL-54 appears to have a defectively-bound (inactive) Mg-ATPase

aggregate (Bragg and Hou, 1973; Maeda *et al*, 1977a), whilst strain NR76 appears to have at least three separate mutations, and its defects are not well-understood (Rosen and Adler, 1975; Adler and Rosen, 1976). Thus it appears that the presence of a correctly-assembled Mg-ATPase aggregate in the membrane is necessary to restrict uncontrolled proton movement. Further evidence for this proposal is given later.

The presence of the Mg-ATPase is also necessary for the maintenance of respiration-linked activities in the membrane. Thus, stripped membranes lack respiration-driven Ca^{2+} transport, which can be restored by the addition of solubilized Mg-ATPase to the membranes (Tsuchiya and Rosen, 1975a, b). A similar effect on the respiration-dependent transhydrogenase was found by Bragg and Hou (1972), although the severity of the defect in stripped membranes varies between strains (cf. Cox *et al*, 1973b; Bragg and Hou, 1973), and appears to be related to the amount of residual energization maintained in the stripped membranes. It should be noted that respiration itself is not greatly affected by the stripping procedure.

The fluorescence of acridine dyes can be used as a monitor of the degree of energization of membrane preparations (Kraayenhof, 1970; Nieuwenhuis *et al*, 1973). The fluorescence of the dye is quenched when the membrane is in an energized state, induced either by respiration or by ATP hydrolysis. Both respiration- and ATP-driven quenching of acridine fluorescence are lost when membranes are stripped of Mg-ATPase, but both activities can be restored in stripped membranes by the addition of Mg-ATPase (Nieuwenhuis *et al*, 1973). The amount of respiration-driven quenching of acridine fluorescence

remaining in stripped membranes varies somewhat (cf. Rosen and Adler, 1975; Hasan and Rosen, 1977; Hasan *et al*, 1978; Nieuwenhuis *et al*, 1973) and appears to depend, like transhydrogenase activity, on the residual energization of the membrane. This technique has proved to be very useful in monitoring membrane energization (Simoni and Postma, 1975) although it is not clear whether the acridine dye distributes solely according to trans-membrane pH gradients, or in response to both pH and the degree of electrostatic interaction of the dye with the membrane (Deamer *et al* 1972; Fiolet *et al*, 1974; Kraayenhof and Fiolet, 1974). In general, however, the occurrence of respiration-linked or ATP-linked activities can be correlated with the occurrence of respiration-driven or ATP-driven quenching, respectively, of acridine-dye fluorescence (Simoni and Postma, 1975; Schairer *et al*, 1976). It appears, therefore, that the presence of the Mg-ATPase is necessary for maximal respiration-driven (as well as ATP-driven) energization of the membrane, monitored either by acridine fluorescence-quenching, or by such parameters as energy-linked transhydrogenase activity or active transport.

It is also apparent that this structural role of the Mg-ATPase complex in respiratory-linked activities can be related to the role of the complex in preventing uncontrolled proton movements, as first proposed by Mitchell (1966). Thus, the mutant strains with membranes leaky to protons, mentioned above, also exhibit defects to varying degrees in respiration-driven active transport, transhydrogenase and acridine fluorescence quenching (Table I.1, strains NR70, DL-54 and NR76).

(f) The mode of action of dicyclohexyl-carbodiimide on the membrane Mg-ATPase

The lipophilic compound dicyclohexyl-carbodiimide (DCCD) has proved a useful tool in studying the role of proton movements in the F_0-sector of the Mg-ATPase. DCCD has been shown to inhibit ATP synthesis and/or breakdown in a variety of bacteria, including *E.coli* (Evans, 1970), *Streptococcus faecalis* (Harold *et al*, 1969), *Mycobacterium phlei* (Kalra and Brodie, 1971; Lee *et al*, 1976), the thermophilic bacterium PS3 (Sone *et al*, 1975), *Azotobacter vinelandii* (Bhattacharyya and Barnes, 1976), *Halobacterium halobium* (Danan and Stoeckenius, 1974), *Clostridium pasteurianum* (Clark and Morris, 1976), and *Chlorobium thiosulphatophilum* (Burns and Midgley, 1976). Moreover, DCCD also affects ATP synthesis and hydrolysis in the chloroplasts of spinach (McCarty and Racker, 1967; Uribe, 1972), and *Euglena* (Porat *et al*, 1976), and in the mitochondria of yeast (Partis *et al*, 1976) and rat heart and beef heart (Beechey *et al*, 1966). However, DCCD as much less effect on Mg-ATPase (F_1) solubilized from membranes of *E.coli* (Roisin and Kepes, 1973), *S. faecalis* (Harold *et al*, 1969), or mitochondria (Roberton *et al*, 1968).

Carbodiimides are rather reactive molecules which react with most of the known functional groups in proteins (Kurzer and Douraghi-Zadeh, 1967) and phospholipids (Bruni *et al*, 1971). However, it is now clear that the lipophilic compound DCCD, at concentrations of 20-100μM, reacts primarily with a single protein ('DCCD-binding protein') in the F_0-sector of the Mg-ATPases of mitochondria (Cattell *et al*, 1971; Stekhoven *et al*, 1972; Partis *et al*, 1976) and *E.coli* (Fillingame, 1975, 1976; Hare, 1975; Altendorf and

Zitzmann, 1975).

Moreover, in *E.coli* there is considerable evidence that DCCD exerts its effects on the Mg-ATPase by binding to this protein, thereby inhibiting proton movement through the F_0-sector of the Mg-ATPase. Thus, DCCD can seal the proton 'leak' in normal membranes stripped of Mg-ATPase (Patel *et al*, 1975; Hasan and Rosen, 1977). In the mutant strain RF-7, which has DCCD-resistant oxidative phosphorylation and Mg-ATPase (Table I.2), DCCD reacts much less readily with the DCCD-binding protein (Fillingame, 1975; Patel and Kaback, 1976). The *dcc-1* mutation in strain RF-7 is more than 90% co-tranducible with the *uncA* gene, strongly suggesting that the *dcc-1* allele affects a gene in the *unc* operon coding for the DCCD-binding protein (Fillingame, 1975). Strains similar to RF-7 have been described by Friedl *et al* (1977) and Altendorf and Zitzmann (1975). Strains NR70, NR76 and DL-54 (Table I.1), as mentioned previously, have membranes which are 'leaky' to protons, and are correspondingly deficient in respiratory-dependent functions. Both the proton impermeability and the respiratory-driven activities can be reconstituted in membranes from these strains simply by the addition of DCCD (Table I.1; Rosen, 1973b; Altendorf *et al*, 1974; Rosen and Adler, 1975). Other strains such as K_{I1} and A_{I44} (Table I.2), N_{I44}, *unc-17* (Table I.1) and AN285 (*unc-405*) (Table I.1; Chapter VII of this thesis) have somewhat similar defects which can be 'cured' by the addition of DCCD. Respiratory-linked activities in stripped membranes of a normal strain can similarly be restored by the addition of DCCD (Nieuwenhuis *et al*, 1973; Tsuchiya and Rosen, 1975a, b; Patel *et al*, 1975; Hasan and Rosen, 1977).

DCCD can thus be considered to mimic the structural role (but not of course the catalytic role) of the Mg-ATPase (F_1) in the membrane, preventing the dissipation of the energized membrane state by uncontrolled proton equilibration across the membrane. Such a mode of action of DCCD in mitochondria was proposed by Racker (1967). The mechanism by which DCCD inhibits proton movement is unknown. It is probable that the reaction site of DCCD is a carboxylic acid group, and that an N-acyl urea is formed (cf. Altendorf and Zitzmann, 1975; Fillingame, 1976). In *E.coli*, the DCCD-binding protein is a hydrophobic protein, soluble in chloroform: methanol, of about 9000 daltons (Fillingame, 1975; Altendorf and Zitzmann, 1975). It has been purified, and its amino-acid composition is remarkably non-polar (Fillingame, 1976; Altendorf, 1977). The DCCD-binding protein is present in a partially-purified F_1-F_0 Mg-ATPase preparation, soluble in the presence of detergent (Hare, 1975). This observation, together with the findings reported above, leaves little doubt that the primary site of action of DCCD is a hydrophobic protein which is an integral part of the membrane Mg-ATPase, and is closely involved in the movement of protons through the F_0-sector of the Mg-ATPase.

(g) Further categorization of *unc* mutant types

Scrutiny of Tables I.1 and 2 reveals that the characterization of many of the mutants isolated is rather incomplete, hampering comparisons between strains. However, it is clear that several of the strains listed in Table I.2 do not fit the accepted pattern of characteristics of *unc* mutants (section (b) above). Most obviously, strains RF-7 (*dcc-1*) (Fillingame, 1975) and DC1 (Friedl *et al*, 1977) (Table I.2), which have altered DCCD-binding proteins as discussed

above, are not uncoupled, since both strains grow at the expense of succinate, indicating that oxidative phosphorylation is intact. Moreover, in strain DC1, ATP-dependent energization of the membrane is unimpaired (Friedl *et al*, 1977). These strains thus have mutations which affect the Mg-ATPase complex, but do not result in any defect, except that of insensitivity to the artificial inhibitor DCCD. Another strain (DG7/1) shown to have a mutation which results in a DCCD-resistant DCCD-binding protein, is apparently uncoupled as well (Altendorf and Zitzmann, 1975). Although it is likely that all these mutations affect the same gene, this has not been shown, and the gene(s) affected have not yet been identified. Two further strains listed in Table I.2 appear to lack oxidative phosphorylation, yet retain some ATP-dependent energization of the membrane. The *etc-15* ('electron-transfer coupling') mutant isolated by Hong and Kaback (1972), retains some ATP-driven transhydrogenase activity (Bragg *et al*, 1973), but fails to grow on the non-fermentable substrates lactate or succinate (Hong and Kaback, 1972). This strain may have several mutations, one of which affects the γ-subunit of the Mg-ATPase but does not affect the ability of the mutant Mg-ATPase to reconstitute stripped membranes (Bragg *et al*, 1973). The *unc-373* mutant described by Thipayathasana (1975), which retains ATPase activity but with altered ion-specificity, exhibits Ca^{2+}-dependent ATP-driven transhydrogenase activity, even though Ca^{2+}- or Mg^{2+}-dependent oxidative phosphorylation is lacking. (The phenotypic classification of this strain as 'UncD' (Thipayathasana, 1975) is not used here, since confusion with the unrelated *uncD* genotype (Cox *et al*, 1978a) would result. Similarly, the *'uncA103c'* designation of Schairer and Haddock (1972) is not used)

(i) ATPase$^-$ *unc* mutant strains

Amongst the *unc* ATPase$^-$ mutant strains, those carrying the *uncA401* allele (Table I.1) most closely-resemble the normal strain in terms of the structure of the Mg-ATPase complex. The inactive Mg-ATPase aggregate solubilized from membranes of *uncA401* mutants is physically indistinguishable from the corresponding normal Mg-ATPase (see Chapter VII; Cox *et al*, 1974; Maeda *et al*, 1976; Bragg and Hou, 1977) except for a marginally-elevated affinity for exogenously-added ADP (Bragg and Hou, 1977). The membrane-sector (F_0) behaves normally with respect to energization before and after stripping, reconstitution with normal Mg-ATPase, and the effect of DCCD (Cox *et al*, 1973a,b; Maeda *et al*, 1977a; Hasan *et al*, 1978). It should be noted, however, that the respiration-dependent transhydrogenase activity is much higher in stripped membranes of an *uncA401* mutant than in the parental strain (Cox *et al*, 1973b), whilst the amount of succinate-induced fluorescence quenching is similar and quite low in such membranes (Hasan *et al*, 1978). These results probably reflect different amounts of residual energization under the conditions of measurement, which would in turn depend on the rates of generation of the energized membrane state by oxidase activity, and of its dissipation through the damaged membrane, as well as through the driving of energy-requiring reactions . (The ATPase activity observed by Gunther and Mariss (1974) and Ahlers *et al* (1976) is due to acetokinase (Bragg and Hou, 1977)).

A group of ATPase$^-$ strains have membranes which are somewhat deficient in their ability to support respiratory-driven activities. The addition of normal Mg-ATPase to the unwashed membranes gives partial reconstitution of both ATP-driven and respiratory-driven functions, but reconstitution is generally better when the membranes are first subjected to the stripping procedure. DCCD

is able to reconstitute respiratory-driven activities in a similar way. Amongst these strains (Table I.1) are: AN285 (*unc-405*) (Chapter VII), DG20/1, BH212, MDA_1, DL-54, NR70 and NR76. The identification of a defect in the unwashed membranes of such strains is often not clear-cut. In unwashed membranes of strains DG20/1 and BH212, for instance, the lactate-driven acridine fluorescence quenching is maximal, whereas the respiration-driven transhydrogenase activity is lower than in normal membranes (Schairer *et al*, 1976). Moreover, in whole cells of these strains, the initial rate of thiomethyl-β-D-galactoside accumulation is about 50% higher than in the parent strain. In the mutant strains, such uptake is completely inhibited by KCN, indicating that it is totally-dependent on respiration (Schairer *et al*, 1976). In strain DL-54, the severe transport defect found in unwashed membranes is not as apparent in whole cells (Berger, 1973; Simoni and Shallenberger, 1972; Altendorf *et al*, 1974). It should be emphasized that the nature of the mutations in this group of strains varies considerably. Thus, strain NR76 has at least three mutations, and strain NR70 may have a deletion or polarity mutation, whereas strain AN285 (*unc-405*) carries a point mutation (see Chapter VII).

Another $ATPase^-$ strain, AS12/25 (Table I.1), can be grouped loosely with the above strains in terms of its properties. In this strain there is come reconstitution of energy-linked activities in unwashed membranes by added Mg-ATPase, but the stripping procedure is actually detrimental to subsequent reconstitution (Schairer *et al*, 1976).

The MDA_2 mutants isolated by Daniel *et al* (1975) appear to form a separate class, in which the binding sites for Mg-ATPase in the F_0-sector, are all available for added Mg-ATPase, even in unwashed

membranes (Table I.1).

Unwashed membranes of strain N_{I44} (Table I.1) have normal respiration-linked transhydrogenase (Kanner and Gutnick, 1972) and transport (Or *et al*, 1973), although acridine fluorescence-quenching is not maximal unless DCCD is added (Nieuwenhuis *et al*, 1973). The stripping procedure has little effect on these membranes, and the addition of normal Mg-ATPase to stripped membranes does not result in reconstitution of ATP-driven activities or respiration-dependent acridine fluorescence quenching, and only low amounts of the added Mg-ATPase bind to the membranes (Nieuwenhuis *et al*, 1973; Bragg and Hou, 1973). Strain N_{I44} lacks the α-, β- and γ-subunits of the Mg-ATPase, and from *in vitro* complementation experiments and the reversion frequency of the mutation, this strain appears to have a point mutation conferring polarity (Nieuwenhuis *et al*, 1973; Kanner *et al*, 1975; Maeda *et al*, 1977a).

Finally, a group of $ATPase^-$ strains have membranes which support respiration-dependent activities as well as normal membranes, but the stripping procedure has no effect on these activities, and normal Mg-ATPase is unable to bind to the stripped membranes or reconstitute ATP-driven activities. Amongst these strains (Table I.1) are: AN463 (*uncD409*) (Chapter VI), BH273, AS69/1 and MDA_3.

(ii) $\underline{ATPase^+}$ *unc* mutant strains

Several of the *unc* mutant strains which retain Mg-ATPase activity, can be grouped together on the basis of the following characteristics: unwashed or stripped membranes support respiration-

driven energization of the membranes, and rebinding of normal Mg-ATPase to stripped membranes does not reconstitute ATP-driven energization. The Mg-ATPase aggregates from the mutant strains are normal, in the sense that they can bind to normal stripped membranes and reconstitute ATP-driven energization. In the mutant membrane *in situ*, however, the Mg-ATPase activity is relatively insensitive to DCCD. Amongst such strains, the best-characterized are (Table I.2); strains carrying the *uncB402* (formerly *uncB401*, Gibson *et al*, 1977a) allele, and strains DG15/10, DG26/4, DG7/10 and DG31/3. In strains carrying the *uncB402* allele, the Mg-ATPase is rather loosely-bound to the membrane, perhaps accounting for its insensitivity to DCCD. Moreover, the Mg-ATPase is de-repressed, and 80% of the activity is found in the cytoplasmic fraction, whilst the amount bound to the membrane is similar to that found in a normal strain (Gibson *et al*, 1977a). The DCCD-binding protein of the *uncB402* mutant has normal reactivity with DCCD (Fillingame, 1975). Strain BG-31 (Table I.2) may also belong to this group of mutants; this strain has an amber mutation in an unspecified *unc* gene, and lacks a protein of 54,000 daltons, which was not the α- or β-subunit of the Mg-ATPase (Simoni and Shandell, 1975).

Strain MDB (Table I.2) has somewhat similar properties to the group of strains just described, but the solubilized Mg-ATPase of strain MDB cannot bind to or reconstitute stripped normal membranes (Daniel *et al*, 1975).

Strains A_{I44} and K_{I1} (Table I.2) also resemble the *uncB402* mutant. They have normal solubilized Mg-ATPase, which can reconstitute acridine fluorescence quenching in stripped normal membranes (Kanner *et al*, 1975). However, the membranes of strains K_{I1} and A_{I44}, unlike

those of the *uncB402* mutant, are unable to support normal levels of respiratory-driven activity (Table I.2). Moreover, although the Mg-ATPase of strains K_{I1} and A_{I44} is insensitive to DCCD, DCCD can restore the respiration-dependent energization of the membrane (Table I.2). As in the *uncB402* mutant, a large proportion of the Mg-ATPase activity is found in the cytoplasmic fraction. Both strains revert to wild-type indicating that they have point mutations (Nieuwenhuis *et al*, 1973).

Strain B_{V4} has a different set of characteristics (Table I.2): unwashed membranes have a lowered capacity to support energization (Nieuwenhuis *et al*, 1973) although transport levels are normal (Kanner *et al*, 1975), and normal Mg-ATPase is unable to bind to stripped membranes of strain B_{V4} or reconstitute energy-linked activities. DCCD is similarly ineffective. Although at least the α-, β- and γ-subunits of the Mg-ATPase are present, the complex cannot reconstitute energy-linked activities in normal membranes. The Mg-ATPase *in situ* is not sensitive to DCCD. The reversion rate indicates that there is a point mutation in strain B_{V4} (Nieuwenhuis *et al*, 1973) which is not in the gene affected in strains K_{I1} and A_{I44}, as deduced by *in vitro* complementation studies (Kanner *et al*, 1975).

Lastly, strains carrying the *uncC424* allele form yet another class of ATPase^{+} mutant (Table I.2). In this strain, there are two types of Mg-ATPase complex. The bulk of the Mg-ATPase activity is found in the cytoplasmic fraction after cell disintegration. This complex is insensitive to DCCD, like other soluble or solubilized Mg-ATPases (Cox *et al*, 1977). However, the cytoplasmic Mg-ATPase of the *uncC424* mutant is slightly inhibited by the lipophilic chelators

bathophenanthroline and tertiary octyl-catechol, whereas the normal solubilized Mg-ATPase (or the *uncB402* cytoplasmic Mg-ATPase) is strongly-stimulated by these compounds (Cox *et al*, 1977). On the other hand, the membrane-bound Mg-ATPase in the *uncC424* strain, like the normal membrane Mg-ATPase, is inhibited by DCCD and the lipophilic chelators (Cox *et al*, 1977). Moreover, in experiments not reported here, it was shown that the membrane-bound Mg-ATPases from the *uncC424* and *unc*$^{+}$ strains had the same mobility during gel electrophoresis under non-dissociating conditions, whereas the cytoplasmic Mg-ATPase had higher mobility than either these species, or the chloroform-solubilized normal Mg-ATPases (see Chapter V), and thus appears to be of lower molecular weight than these other Mg-ATPase complexes (D. Fayle, unpublished experiments).

(iii) Mu-induced polar mutations in the *unc* operon

Recently it was established that four *unc* genes are arranged in an operon, in the order *uncBADC* (Gibson *et al*, 1978). A series of *unc* mutants was obtained after infection of cells with mutator (Mu) phage, which inserts randomly into the chromosome of *E.coli* (Taylor, 1963). If Mu-phage inserts into an operon, it causes a strongly-polar effect on the transcription of genes distal to the point of insertion (see Howe and Bade, 1975). Plasmids containing a known point mutation in the *unc* operon were introduced into each of the uncharacterized Mu-induced *unc* mutants, to form partial diploid strains. The point mutations used were the *uncA401*, *uncB402*, *uncC424* and *uncD409* alleles. The recipient strains used contained the *recA* allele, which prevents recombination between the plasmid and the chromosome. Only the partial diploid strains containing a full complement of normal *unc* genes can

grow on succinate. Thus, if the Mu-induced mutation affects the *uncD* and *uncC* genes, only the *uncB402*, *uncA401*, or *unc*$^+$ alleles will complement the polar mutation, allowing growth of the partial diploid strain on succinate. By such complementation tests, four classes of polar mutants were obtained (Table I.3), and it was deduced that the genes were transcribed in the order *uncBADC*. Energetic properties of the partial diploid strains and of the segregant strains were determined, and those of the segregants are summarized in Table I.3.

All of these Mu-induced mutant strains exhibit the characteristics described in section (b) common to uncoupled strains. Thus the aerobic growth yields are low, and no ATP-driven energization of membranes can be detected. In all cases, the NADH-dependent energization of the membranes is unimpaired. Only one class of strains, *uncB*$^+$*A*$^+$*D*$^+$*C*$^-$, retains Mg-ATPase activity. The absence of at least a portion of the *uncC* gene product thus does not affect the Mg-ATPase activity. Similarly, a point mutation in the *uncC* gene (*uncC424*) does not affect Mg-ATPase activity. However, insertion of Mu-phage in a gene distal to *uncA* but before *uncC* does result in the loss of Mg-ATPase activity (Table I.3). At least the *uncD* gene is present in this region. A further note is that the *uncB*$^+$*A*$^+$*D*$^+$*C*$^-$ class of mutants has an aerobic growth yield on limiting glucose that is somewhat lower than that expected for an *unc* mutant. These strains (like the *uncC424* mutant) have very high levels of Mg-ATPase activity in the cytoplasmic fraction, and the lowered growth yield may be attributable to the lowering of cellular ATP levels in these strains (Gibson *et al*, 1978).

More recently, a further gene, *uncE*, affecting the F_0-sector, has been located between the *uncB* and *uncA* genes, and the number of

TABLE I.3 Bioenergetic properties of strains carrying Mu-induced mutations in the *unc* operon.

The data in this Table are from Gibson *et al* (1978). Gene-designations and transcriptional order were established by genetic and biochemical complementation tests in partial diploid strains containing plasmids carrying known *unc* alleles.

	STRAIN			
BIOENERGETIC PROPERTY	*unc-413*::Mu $uncB^{-}A^{-}D^{-}C^{-}$	*unc-418*::Mu $uncB^{+}A^{-}D^{-}C^{-}$	*unc-421*::Mu $uncB^{+}A^{+}D^{-}C^{-}$	*unc-415*::Mu $uncB^{+}A^{+}D^{+}C^{-}$
LOWERED AEROBIC GROWTH YIELD	+	+	+	See text
Mg-ATPase ACTIVITY	–	–	–	+
ATP-DEPENDENT ATEBRIN FLUORESCENCE QUENCHING	–	–	–	–
ATP-DRIVEN TRANSHYDROGENASE	–	–	–	–
NADH-DEPENDENT ATEBRIN FLUORESCENCE QUENCHING	+	+	+	+

classes of polar mutants is now five, the *uncB*$^{+}$*A*$^{-}$*D*$^{-}$*C*$^{-}$ mutants falling into either the *uncB*$^{+}$*E*$^{+}$*A*$^{-}$*D*$^{-}$*C*$^{-}$ or the *uncB*$^{+}$*E*$^{-}$*A*$^{-}$*D*$^{-}$*C*$^{-}$ classes (J. A. Downie, F. Gibson and G. B. Cox, unpublished work).

The use of Mu-induced *unc* mutants, together with partial diploids of such strains containing plasmids carrying known point mutations, promises to advance knowledge of the biosynthesis of the Mg-ATPase very rapidly. Possible implications of the operon on the stoichiometry of the gene products will be discussed in Chapter X. Moreover, it should be possible to deduce the roles of the various subunits, when the identity of each gene product has been established, from a comparison of the bioenergetic characteristics of point mutants with those of the polar mutants and the partial diploids.

(iv) Antibiotic-resistant *unc* mutant strains

Some of the strains listed in Tables I.1 and 2 were isolated as neomycin-resistant strains unable to grow on non-fermentable carbon sources. It was suggested (Kanner and Gutnick, 1972a) that neomycin resistance could be attributed to the inability of mutant strains to generate sufficient energy to concentrate the antibiotic. Although it was shown by Or *et al* (1973) that whole cells of a derivative of the neomycin-resistant ATPase^{-} mutant N_{I44} (Table I.1) could take up proline or thiomethyl-β-D-galactoside at normal rates, as mentioned above the energization of the membranes was somewhat defective (Nieuwenhuis *et al*, 1973), and transport in vesicles was defective (Van Thienen and Postma, 1973). Several other neomycin resistant mutants (Table I.1 and 2) were defective in active transport, for example K_{I1} (Kanner *et al*, 1975), NR70 (Rosen, 1973a, b; Kobayashi *et al*, 1974; Tsuchiya and Rosen, 1975a, b; Boonstra *et al*, 1975),

NR76 (Rosen and Adler, 1975), and 4 unnamed strains (Yamamoto *et al*, 1973). Adler and Rosen (1976) were able to correlate the degree of neomycin sensitivity with the extent of active transport in strains NR70, NR76, and two 'partial revertants' of the latter strain. Thus it appears likely that neomycin-resistant strains form a special class of *unc* mutants (see also Simoni and Shandell, 1975) defective not only in their energy-linked Mg-ATPase, but also in their ability to support uptake of certain molecules, especially those for which active transport is dependent on the steady-state maintenance of a greater degree of membrane energization than that found in a neomycin-resistant strain (see also Adler and Rosen, 1976).

(h) Suppression of active transport defects in several *unc* mutants by anaerobic growth

It may be deduced from the work summarized in the previous section that the various energy-requiring processes utilize the available energy with different efficiencies, oxidative phosphorylation being the process most severely affected by defective energization. The observations described in this section indicate that the composition of the membrane may affect the efficiency of utilization of energy by a single process, namely active transport.

As noted above, strains (e.g.) *unc-17*, DL-54 and NR70 (Table I.1) were found to have defective active transport, associated with the proton-leakiness of the membranes. Surprisingly, when these strains were grown anaerobically, active transport under anaerobic or aerobic conditions was normal (Yamamoto *et al*, 1973; Boonstra *et al*, 1975; Hasan and Rosen, 1977). Moreover the respiration-dependent

acridine fluorescence quenching was maximal in membranes of strain NR70 grown anaerobically, but low when this strain was grown aerobically (Hasan and Rosen, 1977). Under anaerobic conditions, the membranes of these strains would be rather different to membranes from aerobically-grown cells. In particular, the nitrate reductase was required for both anaerobic growth and active transport (see Konings and Boonstra, 1977).

Moreover, the high levels of respiration-linked active transport in the membranes of strains DL-54 and NR70, grown anaerobically, were not greatly altered by agents which alter proton permeability under aerobic conditions - DCCD or guanidine hydrochloride (Boonstra *et al*, 1975; Hasan and Rosen, 1977). The latter compound is a chaotropic agent which probably strips the F_1 subunits from the membrane leaving at least the Mg-ATPase binding site (Hasan and Rosen, 1977) and the DCCD-binding protein (Patel and Kaback, 1976) *in situ*. Thus guanidine hydrochloride should increase the proton permeability of the 'anaerobic' membranes, as it does that of membranes of aerobically-grown cells (Patel *et al*, 1975), whereas DCCD should prevent proton leakage in the 'anaerobic' membranes of these mutant strains. In neither case does the transport activity respond to the presumed changes in proton permeability.

The resistance to guanidine hydrochloride was related to the presence of the *unc* mutation. In vesicles prepared from the parental strains, grown anaerobically, transport was severely affected by prior treatment with guanidine hydrochloride, although proline transport (Boonstra *et al*, 1975) appears to be considerably more susceptible to such treatment than Ca^{2+} transport (cf. Hasan and Rosen, 1977).

Anaerobic growth of the *unc* mutant strains DL-54 and NR70, therefore, resulted in more effective electron-transfer-driven energization of the membranes, under conditions in which the proton-leakiness of the membranes might be expected to dissipate the energized membrane state. Unfortunately, measurement of the rate of proton equilibration across membranes from anaerobically-grown cells presents technical difficulties (Hasan and Rosen, 1977), and so it is not yet clear whether in these membranes, there is a reduced rate of dissipation of energy through the altered Mg-ATPase aggregate, or alternatively more rapid (and thus more efficient) utilization of generated energy, than in membranes from aerobic mutant cells. In either case, the role played by the altered composition of the membrane must be considered in any comparison of energy-linked activities or proton-permeability of mutant strains.

When the F_0-sector (TF_0) of the ATPase from the thermophile PS3 was purified and reconstituted into vesicles, it allowed the passive permeation of protons across the vesicle membranes (Okamoto *et al*, 1977). Interestingly, the rate of such permeation was largely dependent on the fluidity of the phospholipids around the TF_0 (Okamoto *et al*, 1977). The fluidity in turn is dependent on the fatty-acid composition of the lipids (Wilson and Fox, 1971; Träuble and Overath, 1973; Overath and Träuble, 1973) and on the ionic environment of the phospholipid head groups (Träuble and Eibl, 1974). One or both of these properties could conceivably account for the anaerobic effect discussed above, and the effect of ions could also be responsible for discrepancies observed in the energization of various processes in mutants with elevated proton permeability, measured in different buffers under different conditions.

(i) Other mutations affecting the activity of the Mg-ATPase

A mutation in strain AN295 (Cox *et al*, 1973a; Butlin, 1972) resulted in the de-repression of Mg-ATPase activity, and also in the failure of the strain to grow on succinate unless adenine and either serine or methionine were also added. Spermine or S-adenosyl-methionine also suppressed the Suc^- phenotype (Butlin, 1972). The mutation maps about 4 min away from the *unc* operon, and the nature of the gene product is not known.

Finally, a series of *ecf* ('energy-coupling factor') mutants have been isolated by Hong and co-workers (Lieberman and Hong, 1974; Lieberman *et al*, 1977; Hong, 1977; Tomochika and Hong, 1978). The mutants map at min 64, in or near the *metC* gene, the structural gene for the enzyme cystathionase which is involved in methionine biosynthesis (see Bachmann *et al*, 1976). The lesions in these strains fall into three classes. Strain MAL300 is unable to maintain the energized membrane state, but the membranes are impermeable to protons (Lieberman *et al*, 1977). On the other hand, strain JSH270 is able to maintain the energized membrane state, but cannot use it to drive active transport (Hong, 1977). A third type of mutant, strain JSH267, is leaky to a number of small ions, including protons, and is thus unable to maintain the energized membrane state or support transport (Tomochika and Hong, 1978). The relationship between the *ecf* gene product and energy-coupling, however, is not as yet clear.

It is interesting that methionine is required, as a donor of methyl groups, for the energization of chemotaxis in *E.coli* (Kort

et al, 1975; Springer *et al*, 1975). ATP is also required, but the Mg-ATPase is not, and it has been suggested that S-adenosyl methionine may be involved (Larsen *et al*, 1974; Adler, 1975; Koshland, 1977). Clearly, further characterization of the role of ATP and methionine metabolites in energy transduction is required, particularly in the light of evidence that some active transport systems are energized by ATP in *unc* mutants (Berger, 1973; see also Simoni and Postma, 1975; Hamilton, 1977; Harold, 1977a).

C. THE STRUCTURE OF THE MEMBRANE Mg-ATPase

There is now a very large body of published work on the structures of Mg-ATPases from many organisms, and this work has been reviewed in depth by Senior (1978, 1973), Haddock and Jones (1977), Sebald (1977), Panet and Sanadi (1976), Nelson (1976), Pedersen (1975) and Simoni and Postma (1975). As mentioned before, the membrane complex can be considered as an F_1-F_0 complex, with the F_0-sector containing the binding-site for the F_1-complex, which is solubilized more easily. Although the term 'F_1' serves well as a working designation, caution must be applied in the use of 'F_1' in the light of recent evidence suggesting the involvement of proteolytic activity in the solubilization of the F_1 Mg-ATPase from *E.coli* membranes (see Chapters IV and V; Cox *et al*, 1978b). Nevertheless, the F_1 Mg-ATPase, once solubilized, can bind back to the F_0-sector in the stripped membranes, reconstituting normal functions.

(a) Purification of the F_1-portion of the Mg-ATPase

A wide variety of methods have been used to purify the F_1-portion of the Mg-ATPase. The methods in current use are listed in Senior (1978). In *E.coli*, the Mg-ATPase is normally solubilized by washing the membranes with buffers of low-ionic strength in the absence of Mg^{2+} (see Chapter IV; Bragg and Hou, 1972; Roisin and Kepes, 1973; Cox *et al*, 1973a, 1978b). Various methods have been used for subsequent purification of the complex, including fractionation using polyethyleneglycol (Vogel and Steinhart, 1976) or ammonium sulphate (Nelson *et al*, 1974), ion-exchange chromatography (e.g. Bragg and Hou, 1972; Kobayashi and Anraku, 1972; Hanson and Kennedy, 1973; Nelson *et al*, 1974; Futai *et al*, 1974), gel filtration (e.g. Bragg and Hou, 1972; Cox *et al*, 1973a, 1974; Nelson *et al*, 1974; Futai *et al*, 1974; Vogel and Steinhart, 1976), and sucrose or glycerol density gradient centrifugation (e.g. Bragg and Hou, 1972; Hanson and Kennedy, 1973; Kobayashi and Anraku, 1974; Nelson *et al*, 1974). Some other methods have been used for the purification of bacterial Mg-ATPases, notably affinity chromatography on agarose gels linked to an ATP analogue (Hulla *et al*, 1976) or to ADP (Higashi *et al*, 1975).

(b) Structure of the F_1-portion of the Mg-ATPase

The F_1-portion of the Mg-ATPase has a molecular weight of around 360,000 in *E.coli*. The molecular weight values obtained by various workers, together with the methods used to estimate them, are listed in Chapter V (see Table V.1). The F_1-ATPases from other organisms have approximately the same molecular weight, although they are not all identical (Adolfsen *et al*, 1975; Senior, 1978).

The F_1-portion of the *E.coli* Mg-ATPase contains five types of subunit, designated α, β, γ, δ, ε, in order of decreasing molecular weight. The approximate molecular weights for the subunits are: α, 54,000; β, 48,000; γ, 30,000; δ, 20,000; ε, 12,000. The values obtained by various workers are also given in Chapter V (see Table V.2). In all but one instance, the molecular weights were estimated by gel electrophoresis in the presence of sodium dodecyl sulphate (SDS). Again, in other organisms, the Mg-ATPase subunit composition resembles that in *E.coli*, although the minor subunits vary somewhat (Haddock and Jones, 1977; see also Chapter IX).

(c) Stoichiometry and arrangement of the subunits of the F_1-portion of the Mg-ATPase

There is a considerable amount of controversy over the stoichiometry of the Mg-ATPase subunits (see Senior, 1978). The most likely stoichiometries for the α: β: γ: δ: ε subunits in *E.coli* are 2:2:2:2:2 or 2:2:2:1:2 (Vogel and Steinhart, 1976) or 3:3:1:1:1 (Bragg and Hou, 1975). The former ratios were obtained on the basis of the reassociation of the (α, γ, (δ), ε) and (β) fragments obtained by cold dissociation of the solubilized Mg-ATPase (Vogel and Steinhart, 1976), whilst the latter value was estimated after radioactive-labelling of *E.coli*, followed by SDS electrophoresis and radioactive analysis of the gel (Bragg and Hou, 1975). Stoichiometries obtained by the latter method are rather inconclusive (cf. Kagawa *et al*, 1976).

In the light of the similarities between Mg-ATPases from various organisms, it is very likely that the subunits are arranged similarly in the intact aggregates. However, various interpretations

made from chemical cross-linking data are in conflict, preventing any conclusions as to the arrangement of subunits (Senior, 1978; Bragg and Hou, 1976). Individual subunits purified from the thermostable Mg-ATPase complex of the thermophilic bacterium PS3 were incubated together in various defined mixtures. A ($\beta + \gamma$) complex was shown to be formed, and moreover to have regained Mg-ATPase activity (Yoshida *et al*, 1977a). The ($\alpha + \gamma$) complex, however, did not appear to form (Yoshida *et al*, 1977a). Thus the β- and γ-subunits are probably linked *in vivo*. These workers subsequently presented evidence that the δ- and ε-subunits can each bind to the TF_0-sector, but that the binding of the γ-subunit (or the $\alpha + \beta + \gamma$ complex) was largely dependent on the presence of both the δ- and the ε-subunits on the membrane (Yoshida *et al*, 1977b). Thus it is probable that there are also γ-δ and γ-ε links *in vivo*. Clearly, more information is needed before the juxtaposition of subunits in the Mg-ATPase can be determined.

(d) Functional roles of the subunits of the F_1-portion of the Mg-ATPase

There are a number of indications that a catalytic site for ATPase activity resides in the β-subunit(s). Trypsin treatment of the Mg-ATPase from *E.coli* leaves a complex thought to consist only of ($\alpha + \beta$) which retains activity (Nelson *et al*, 1974). (However, it should be noted that the individual α- and β-subunits are apparently incapable of assembling into such a complex (Yoshida *et al*, 1977a; cf. Larson and Smith, 1977)). Secondly, the ATPase-inhibitor 7-chloro-4-nitrobenzo-2-oxa-1, 3-diazole (NBD-Cl) reacts with a single tyrosine residue in the β-subunit of the Mg-ATPases of mitochondria (Ferguson *et al*, 1975) and *E.coli* (Bragg and Hou, 1977). Thirdly, the antibiotic

aurovertin, which inhibits ATP synthesis (Roberton *et al*, 1968), binds to the β-subunit of the Mg-ATPases of yeast (Douglas *et al*, 1977) and beef heart (Verschoor *et al*, 1977) mitochondria. Fourthly, reconstituted complexes containing the (β + γ) or (α + β + δ) subunits, regained Mg-ATPase activity (Yoshida *et al*, 1977a), and β is the only subunit common to these complexes. Perhaps more than one β-subunit is needed for a catalytic site; or the presence of either the γ or the (α + δ) is necessary to stabilize the conformation of an active site in the β-subunit.

Evidence for the separate binding of the δ- and ε-subunits to the F_0-sector has already been discussed. The δ-subunit has often been invoked as the 'stalk' polypeptide, but the logic behind this conclusion is not tight, as pointed out later (Chapter V, section F).

A sixth F_1-ATPase component in the complex solubilized from beef heart mitochondria is the "ATPase-inhibitor", a basic protein which inhibits the Mg-ATPase activity of membrane-bound F_1-F_0 or soluble F_1, but does not affect ATP synthase activity (Pullman and Monroy, 1963; Van de Stadt *et al*, 1973; Ferguson *et al*, 1977). The evidence that the ε-subunit plays a similar role in *E.coli* (Nieuwenhuis *et al*, 1974b; Smith *et al*, 1975; Sternweis and Smith, 1977; Nieuwenhuis and Bakkenist, 1977) is rather tenuous (see Chapter V, section F).

Clearly, much is yet to be learned about the roles of the subunits in the various functions of the Mg-ATPase complex.

(e) Structure and Composition of the membrane sector (F_0-portion) of the Mg-ATPase

Little is known about the structure of the F_0-sector of the Mg-ATPase. The DCCD-binding protein already described is one component. The interesting possibility that the DCCD-binding protein may form trimers *in vivo* has been raised by Fillingame (1976) and Altendorf (1977) on the basis of radioactive-labelling of the protein with (^{14}C)-DCCD.

The *uncB* gene product could well be a separate component in the F_0-sector, since the reactivity of the DCCD-binding protein of the *uncB402* mutant to DCCD is normal (Fillingame, 1975). The identity of the *uncC* and *uncE* gene products, which may also affect the F_0-sector (Gibson *et al*, 1977b, unpublished work), has not yet been established.

It should be noted that other proteins or 'factors' are present in the F_0-sector of the mitochondrial Mg-ATPase, notably the oligomycin-sensitivity-conferring protein, factor "B", and F_6 (Senior, 1978, 1973; Beechey and Cattell, 1973). It is not yet known whether analogous proteins are present in bacterial Mg-ATPases.

(f) Preparations of the F_1-F_0 Mg-ATPase complex in a dispersed form soluble in detergent

Preparations of the F_1-F_0 Mg-ATPase complex from *E.coli* have been obtained by Nieuwenhuis *et al* (1974a), Hare (1975), Bragg and Hou (1976) and Friedl *et al* (1977). All of these preparations appear to be rather impure, the best-characterized being those of Hare (1975) and Bragg and Hou (1976), which contain rather large amounts of unidentified

high molecular weight proteins. The complex isolated by Hare also had clearly-identified F_0 components, of 29,000 and 9,000 daltons, the latter one being the DCCD-binding protein. The F_1-F_0 Mg-ATPase isolated by Bragg and Hou (1976) had at least 11 components other than the F_1-subunits, including two resembling those in the complex of Hare (1975).

The DCCD-sensitivity of the Mg-ATPase in such complexes was rather low, unless phospholipids were added (Friedl *et al*, 1977), and it has been shown that phospholipids are necessary for oligomycin- or DCCD-sensitivity in such complexes from other organisms (e.g. Ryrie, 1975a; Stiggall *et al*, 1978; Berden and Voorn-Brouwer, 1978). As noted above, phospholipid fluidity regulates proton flux through the F_0-sector (Okamoto *et al*, 1977), and the DCCD-binding protein has been implicated in the process of proton conduction (see section B(f)). It is not clear whether a vesicular structure is required for the action of DCCD. Evidence that the "boundary-lipid" is involved in the structure and function of mitochondrial Mg-ATPase is summarized in Senior (1978). Phospholipids have been detected in the F_1 Mg-ATPase of *E.coli* (Peter and Ahlers, 1975), and are present in the partially-purified F_1-F_0 Mg-ATPase preparations mentioned above.

The best-characterized F_1-F_0 ATPase complexes are those from yeast mitochondria (see Chapter IX), beef heart mitochondria (Serrano *et al*, 1976; Stiggall *et al*, 1978; Berden and Voorn-Brouwer, 1978) and the thermophile PS3 (Sone *et al*, 1975; Kagawa *et al*, 1976; Yoshida *et al*, 1977a,b). The latter is the best characterized bacterial Mg-ATPase, and furthermore has the unusual and invaluable properties that it is stable to high temperatures and high concentrations of denaturing agents (Yoshida *et al*, 1975), and that the isolated

TF_1-subunits are stable, and TF_1 can be renatured from them without added nucleotides (Yoshida *et al*, 1977a, b; Ohta *et al*, 1978).

Notable similarities between most of the mitochondrial F_1-F_0 ATPase complexes isolated include the presence of three presumptive F_0 proteins of about 25,000, 23,000 and 21,000 daltons, and three more between 15,000 and 8,000 daltons. The F_0-sector from the thermophilic bacterium, however, appeared to be rather different, having three components of 19,000, 13,500 and 5,400 daltons (Kagawa *et al*, 1976) although a densitometric trace (Okamoto *et al*, 1977) suggests that TF_0 components analagous to those present in mitochondria (see above) could well be present in the thermophile, together with a component of about 65,000 daltons, resembling that found in yeast F_1-F_0 by Ryrie and Gallagher (see Chapter IX) and in beef heart preparations by Berden and Voorn-Brouwer (1978) and Stiggall *et al* (1978). The F_0-sector has apparently been purified from beef-heart mitochondria by Ernster's group (see Ernster *et al*, 1977). Finally, it should be noted that the 'Complex V' of Stiggall *et al* (1978) was soluble in aqueous media without detergent, and although phospholipids were necessary for complete expression of activities such as ATPase and ATP-P_i exchange, the complex did not appear to be in vesicles.

The use of biochemical genetics provides a most promising approach towards the determination of the number and kind of F_0-subunits in *E.coli*. As pointed out before, such an approach facilitated the identification and purification of the DCCD-binding protein (Fillingame, 1975, 1976; Hare, 1975; Altendorf and Zitzmann, 1975; Altendorf, 1977). However, the use of the stable TF_1-F_0 ATPase from the thermophilic bacterium, and of the purified TF_0-sector, offers an attractive alternative approach, which, together with the genetic

approach, should lead rapidly to the elucidation of many problems in the field.

D. THE FUNCTIONS OF THE MEMBRANE Mg-ATPase

It is now well-established (see section F below) that F_1-F_0 Mg-ATPase complexes, purified and reassembled into vesicles, can catalyse the phosphorylation of ADP in response to energy, supplied by light + bacteriorhodopsin, by artificial electron-transport chains, or by the imposition of a pH gradient by 'pH jump' experiments (Yoshida *et al*, 1975; Ryrie and Blackmore, 1976; Sone *et al*, 1977b). In *E.coli*, ATP synthesis has been demonstrated in vesicles prepared from native membranes, in response to oxidation (Butlin *et al*, 1971; Hertzberg and Hinkle, 1974), or to an artificially-induced electrochemical gradient (Tsuchiya and Rosen, 1976; Tsuchiya, 1977).

It is equally clear from results already described that the F_1-F_0 Mg-ATPase can catalyse ATP hydrolysis in such a way that the membrane is energized. ATP-induced energization, monitored by the enhancement of fluorescence of 1-anilinonaphthalene-8-sulphonate (ANS), has been measured in vesicles containing purified TF_1-F_0 (Sone *et al*, 1975, 1977a; Kagawa *et al*, 1976). Similarly, ATP-induced acridine fluorescence quenching was obtained in vesicles containing TF_1-F_0 (Yoshida *et al*, 1975), or purified F_1-F_0 ATPase from beef heart mitochondria (Berden and Voorn-Brouwer, 1978). Proton translocation, coupled to ATP hydrolysis (see below), has been observed in vesicles containing purified yeast mitochondrial F_1-F_0 ATPase (Ryrie and Blackmore, 1976), or beef heart mitochondrial F_1-F_0 ATPase (Serrano

et al, 1976).

The basic functions mentioned above are probably common to Mg-ATPases in all organisms. In *E.coli*, the Mg-ATPase also serves a more specialized function, that of the ion-receptor involved in chemotaxis in response to divalent cations (Koshland, 1977). Free-living *E.coli* move by 'smooth-swimming' and 'tumbling', combined into a 'random walk'. The organism is able to respond to changes over time in the concentration of nutrients, ions and toxins, by biasing the random walk in the required direction. The bacterium senses a favourable change in concentration, and responds by swimming smoothly. If the gradient is also a spatial gradient, the resulting bias is in the most favourable direction. The attractant, repellent, or ion binds to a receptor: in the case of Mg^{2+}, Ca^{2+}, Zn^{2+}, Mn^{2+} and Co^{2+}, the receptor involved is the Mg-ATPase (Zukin and Koshland, 1976; Koshland, 1977). The ions enter the cell by passive diffusion and/or several transport systems (Koshland, 1977). The mechanism of taxis in response to ions appears to involve specifically ATPase activity, since a strain carrying the *uncA401* allele gives no response to these divalent cations (Zukin and Koshland, 1976). The ATPase activity may not need to be coupled, since the *unc* strain BG-31 (Table I.2) exhibits normal taxis in response to these ions (Zukin and Koshland, 1976). Taxis in response to other attractants and repellents does not require the ATPase, since this function is unaffected in an *uncA401* mutant (Zukin and Koshland, 1976). However, such chemotaxis appears to require ATP, together with methionine, and, as mentioned before, S-adenosyl methionine has been implicated in the process (Adler, 1975; Larsen *et al*, 1974; Koshland, 1977). Motility in general depends on the energization of the cytoplasmic membrane via electron-transport (Larsen *et al*, 1974),

or presumably also ATP hydrolysis (cf. Thipayathasana and Valentine, 1974).

E. PROPERTIES OF THE Mg-ATPase COMPLEX

(a) Nucleotide and metal ion specificity of the Mg-ATPase from *E.coli*, and the effects of pH and ions

The optimum pH for Mg-ATPase activity of the solubilized (F_1) Mg-ATPase from *E.coli* was about pH 9 to 9.5 (Kobayashi and Anraku, 1972; Davies and Bragg, 1972; Roisin and Kepes, 1973). The enzyme requires a divalent cation, normally Mg^{2+}, but Ca^{2+}, Zn^{2+}, Mn^{2+} or Co^{2+} could also fulfil the requirement (Evans, 1969; Kobayashi and Anraku, 1972). The order of cation specificity is similar to that observed for ion chemotaxis (Zukin and Koshland, 1976). The optimum ratio of Mg^{2+} : ATP is about 2:5 (Evans, 1969; Kobayashi and Anraku, 1972). For nucleoside triphosphatase activity, specificity was not very tight: dATP, GTP, and ITP were also substrates (Kobayashi and Anraku, 1972; Davies and Bragg, 1972; Hanson and Kennedy, 1973). UTP and pyrophosphate were hydrolysed very slowly, whilst very little CTPase or ADPase activity could be detected (Kobayashi and Anraku, 1972). For ATPase activity, the K_m (ATP) is about 0.6mM and the K_i (ADP) is about 0.3mM (Kobayashi and Anraku, 1972). However, Kozlov and Skulachev (1977), in reviewing the role of Mg^{2+} in ATP hydrolysis, concluded that Mg.ATP was the true substrate, and that free ATP was a competitive inhibitor (in mitochondria, K_i = 8mM).

The interactions of cations and anions with the membrane-

bound and soluble ATPases are rather complex, and moreover, depend on the pH, the divalent cation:ATP ratio, and the concentrations of the products. The interactions have been investigated in great detail by Günther *et al* (1974) and Ahlers and Günther (1975a, b), and also Evans (1969), Roisin and Kepes (1972) and Carriera and Muñoz (1975). The presence of other enzyme activities which are significant under certain conditions (e.g. acetokinase, see Bragg and Hou, 1977) is a complication, but it is probable that monovalent cations compete with Mg^{2+}, inhibiting Mg-ATPase activity, and that the degree of inhibition by anions is according to the chaotropic series: $SCN^- > NO_3^- > I^- > Cl > HCO_3^- >$ acetate$^-$ (Günther *et al*, 1974).

(b) Nucleotide binding sites

The Mg-ATPase has a rather complicated set of binding sites for nucleotides, and recent reviews by Senior (1978) and Harris (1978) have summarized the information available. The nucleotide binding sites of the enzyme from *E.coli* (Maeda *et al*, 1976, 1977b; Bragg and Hou, 1977) appear to be similar to those of other organisms (Harris, 1978; Senior, 1978), and therefore the conclusions of these reviewers will be outlined briefly. There are three detectable types of nucleotide binding site: the catalytic site for ATP hydrolysis (already mentioned), equilibrium binding sites (K_d 1-5 x 10^{-6}M), and tight-binding sites (K_d < 10^{-8}M).

The affinity of the tight-binding sites for nucleotides is very high (K_d < 10^{-8}M) in the solubilized F_1-portion of the Mg-ATPase, or in the membrane form of the enzyme under de-energized conditions, but a portion of these sites are somewhat less tight (K_d about 5 x 10^{-6}M) when the membrane is energized, and the nucleotide becomes

exchangeable. There are about four tight-binding sites for ADP and ATP in the solubilized Mg-ATPase. Tight-binding sites cannot accept GTP, ITP or other ATP-analogues, even though these molecules are hydrolysed, indicating that the tight-binding sites are not specifically-involved in ATP hydrolysis by the F_1-ATPase. Moreover, removal of the tightly-bound nucleotide did not affect ATP hydrolytic activity, although it did prevent coupling of such hydrolysis to energization of a reconstituted membrane.

The enzyme has one or two sites, specific for ADP, which are not "tightened" during de-energization. Nucleotides bound to these sites remain exchangeable, and the K_d of the sites (around 10^{-5}M) resembles that of the energized "tight-binding" sites. These are known as "equilibrium" binding sites.

Thirdly, there is the site at which ATP can be hydrolysed, which binds nucleotides relatively loosely. This site is also much less specific for nucleotides, as already mentioned.

Lastly, there appears to be regulatory site(s) in which bound nucleotides can regulate the ATPase activity. These may be also tight-binding sites, in which the nucleotide remains non-exchangeable during energization. Recent work has suggested that the conformation of the solubilized TF_1-ATPase depends on the nucleotide bound at an unspecified site (Ohta *et al*, 1978).

The involvement of the various nucleotide binding sites in current schemes for coupled ATP synthesis and hydrolysis will be discussed in section (F) below.

(c) Conformational changes in the Mg-ATPase complex

There is now strong evidence that substantial conformational changes in the Mg-ATPase protein occur during the processes of ATP synthesis and hydrolysis. Ryrie and Jagendorf (1972) showed that, on energization, the chloroplast Mg-ATPase complex transiently sequestered (^3H)-H_2O, and that such incorporation was prevented by inhibitors of photophosphorylation, or by uncouplers, which dissipate the energized state of the membrane. Aurovertin, which as already pointed out, binds to the β-subunit of the mitochondrial Mg-ATPase, alters its fluorescence according to the conformation of the Mg-ATPase, which in turn, depends on the energization of the membrane. Energization of the chloroplast Mg-ATPase exposes previously-inaccessible groups to N-ethylmaleimide, sulphate, and several other reagents. The evidence implicating conformational changes in the behaviour of these reagents, and of aurovertin, has been summarized by Senior (1978).

The most direct evidence for conformational changes of considerable magnitude, is that the "tightly-bound", and presumably inaccessible, nucleotides, become rapidly exchangeable on energization of the membrane, and the exchange is sensitive to uncouplers (Senior, 1978).

F. THE RELATIONSHIP OF ATP SYNTHESIS AND HYDROLYSIS TO THE ENERGIZED STATE OF THE MEMBRANE

The mechanisms operating during ATP synthesis, dependent on a high-energy state of the membrane, and ATP hydrolysis, coupled to the

generation of such a high-energy state, have been the subject of vigorous debate over the last 25 years. Although the argument in the past has been clouded by misunderstandings and entrenched opinions, some semblance of agreement is now tending to emerge amongst workers in the field (Boyer, Chance, Ernster, Mitchell, Racker, and Slater, 1977) although this is by no means universal (e.g. Ferguson, 1977; Williams, 1978). Recent proposals for the mechanisms of ATP synthesis and hydrolysis are described in the references mentioned above, as well as Mitchell (1976, 1977), Harris (1978), Senior (1978), Kayalar *et al* (1977) and Kozlov and Skulachev (1977).

(a) The overall reaction: reversible ATP hydrolysis

The overall reaction can be stated briefly as:

$$ATP^{4-} + H_2O \rightleftharpoons ADP^{3-} + PO_4^{3-} + 2H^+ \qquad \text{Equation (1)}$$

It should be noted, however, that the state of protonation of ATP, ADP and P_i will not be simply as depicted, and that any mechanism must take this into account (see Mitchell, 1977). The notion of the "reversibility" of the reaction has been complicated by semantics (cf. for example, Mitchell, 1977 and Williams, 1978), but there are also more fundamental objections to the assumption that the ATPase *in vivo* acts in either direction (Ferguson, 1977). The latter author has reviewed evidence that the ATPases of mitochondria and of obligately aerobic bacteria such as *Paracoccus denitrificans*, act only in the direction of synthesis *in vivo*. The ability of the Mg-ATPase in *E.coli* to catalyse ATP hydrolysis *in vivo* is envisaged as being necessary for adaptation to fermentative metabolism (Ferguson, 1977). Indeed, most of the energy-requiring reactions in *E.coli* (under normal

aerobic conditions) appear to be supportable solely by electron transport, for instance, motility (Larsen *et al*, 1974), transhydrogenase activity (Cox *et al*, 1973b) and active transport of certain molecules (Schairer and Haddock, 1972; Simoni and Postma, 1975; Harold, 1977a). It is most likely, therefore, that the Mg-ATPase acts mainly in the direction of synthesis under these conditions.

(b) Proton-pumping linked to ATPase or synthase activities

In previous sections of this Chapter, the evidence for a proton channel in the F_0-sector of the Mg-ATPase was discussed. This channel serves to carry protons passively across the membrane in the absence of the F_1-portion of the Mg-ATPase. There is now substantial evidence that the Mg-ATPase has an active role as a proton translocase, linked to ATP synthesis and hydrolysis, as proposed by Mitchell (see Mitchell, 1966). Protons are translocated from the F_1 side of the membrane to the opposite side during ATP hydrolysis, and in the reverse direction during ATP synthesis, through the channel in the F_0-sector. *In vitro*, proton translocation from one aqueous phase to the other is well-documented (Mitchell, 1976; Harold, 1977a), although the situation *in vivo* is not as clear-cut, as will be discussed below.

Proton uptake occurs as a result of ATP hydrolysis in vesicles containing purified F_1-F_0 ATPase (oriented outwards) from beef heart (Serrano *et al*, 1976) and yeast (Ryrie and Blackmore, 1976) mitochondria, as measured directly. Non-specific ATP hydrolysis complicates such measurements (West and Mitchell, 1974; Yoshida *et al*, 1975). Proton uptake has been inferred from the ATP-dependent enhancement of

1-anilinonaphthalene-8-sulphonate (ANS) fluorescence in similar vesicles containing the thermostable bacterial TF_1-F_0 complex (Sone *et al*, 1975, 1976). The quenching of acridine fluorescence was used similarly (Yoshida *et al*, 1975; Sone *et al*, 1976). However, as pointed out by Williams (1978), such chemicals may cause an artificial equilibrium between any changes contained in the membrane, and the aqueous phase, and so indirect methods of measurement of charge and proton movement must be interpreted with due caution. In inverted vesicles of cytoplasmic membrane from *E.coli*, proton uptake linked with ATP hydrolysis has been measured directly (West and Mitchell, 1974; Hertzberg and Hinkle, 1974; Hasan *et al*, 1978) and is lacking in membrane vesicles from *uncA401* and *uncB402* mutant strains (Hasan *et al*, 1978), indicating that ATP hydrolysis *via* the Mg-ATPase is involved, and that the hydrolysis must be coupled to membrane energization for proton uptake to occur.

In 'mosaic' vesicles of *E.coli* cytoplasmic membranes, in which the Mg-ATPase faces inwards, transient ATP synthesis has been observed as a result of proton influx, produced by a 'pH jump' experiment, in which vesicles equilibrated at pH 8.2 were placed into a buffer at pH 2.5 (Tsuchiya and Rosen, 1976). Such ATP synthesis was first observed, in chloroplasts, by Jagendorf and Uribe (1966).

(c) The protonic potential difference: its involvement in ATP synthesis and its generation by ATP hydrolysis

The chemiosmotic hypothesis of Mitchell (1966, 1976, 1977 *and in* Boyer *et al*, 1977) equates the 'energization' of the membrane to the total protonic or proton-motive potential difference (Δp) across

the insulating membrane. This quantity consists of an electrical potential difference across the membrane ($\Delta\psi$) and a chemical potential difference across the membrane, which is a function of the concentration difference of protons (ΔpH). These quantities are not independent - the translocation of an H^+ ion across the membrane alters both the ΔpH and the $\Delta\psi$. Conversely, the combination of ΔpH and $\Delta\psi$ exerts a 'proton-motive force' (Δp) (which is the protonic potential difference).

The evidence that energization can be represented by the protonic potential difference has been reviewed in general by Mitchell (1976) and, for bacteria in particular, by Harold (1977a) and Haddock and Jones (1977). The translocation of protons linked with ATP hydrolysis generates a protonic potential difference. Evidence for the involvement of a ΔpH was listed above, and the following observations implicate the proton-motive force (Δp) in the energization *in vitro* of ATP synthesis.

In vesicles containing purified thermostable bacterial TF_1-F_0 ATPase, oriented outwards, synthesis of ATP from ADP and P_i has been measured after the imposition of an artificial proton-motive force, generated by an acid-alkali jump in the presence of K^+ ions and the K^+ ionophore, valinomycin (Sone *et al*, 1977b). In the 'mosaic' membrane vesicles from *E.coli*, mentioned above, ATP synthesis has been demonstrated by a similar method, using an alkali-acid pH jump in the presence of valinomycin + K^+ ions (Tsuchiya, 1977; Tsuchiya and Rosen, 1976). In whole cells of *E.coli*, oxidative phosphorylation, glycolysis, and other metabolic reactions present problems in the measurement of ATP synthesis in response to such an artificial proton-motive force. However, several workers have obtained ATP synthesis in whole cells of

E.coli which is clearly energized by the artificial proton-motive force, and is not detected in several *unc* mutants (Maloney *et al*, 1974; Grinius *et al*, 1975; Wilson *et al*, 1976). ATP synthesis in response to an artificial Δp has also been investigated in whole cells of the bacterium *Streptococcus lactis*, a strict anaerobe, thought to have no electron-transport chain and thus no complicating oxidative phosphorylation (Maloney, 1977). The conclusion was drawn that there was a threshold Δp, below which protons penetrated into the cell slowly. Above the threshold protonic potential, however, proton entry was more rapid, and was coupled with ATP synthesis, which was also sensitive to DCCD. Glycolysis had been blocked with iodoacetate (Maloney, 1977). It should be noted, however, that electron-transfer chains have so far been identified in a number of strict anaerobes, and that in membranes from *Streptococcus faecalis*, the reduction of fumarate by NADH can be coupled with ATP synthesis (see Konings and Boonstra, 1977). It is not known whether such bacteria use energy derived from electron-transport to synthesize ATP anaerobically. Yet another means of generating an artificial Δp, that of imposing a $\Delta\psi$ by means of an applied electrical field, has been shown to energize ATP synthesis in chloroplasts (Witt *et al*, 1976).

It is clear from these and other observations that a protonic potential difference of the type proposed by Mitchell (1966, 1976) can energize ATP synthesis. Such a protonic potential difference is considered to be generated during electron-transport, or during light-induced proton translocation via bacteriorhodopsin (see Oesterhelt *et al*, 1977; Harold, 1977a), although measurement of Δp presents a problem, and is normally done by indirect methods. Ramos *et al* (1976), for instance, measured the ΔpH by the distribution of weak acids across

the membrane, and the $\Delta\psi$ by the distribution of a lipophilic cation. A comment by Williams (1978), already mentioned, is that chemicals may cause the equilibration of charge between the membrane and the aqueous phase. Nevertheless, the protonic potential difference is still presumably present, in some form.

In vesicles containing purified F_1-F_0 ATPase and cytochrome oxidase from beef heart mitochondria, ATP synthesis occurred in response to electron-transport from cytochrome c to oxygen (Serrano *et al*, 1976). Vesicles containing F_1-F_0 ATPase and bacteriorhodopsin could catalyse ATP synthesis on illumination (Yoshida *et al*, 1975; Ryrie and Blackmore, 1976). An artificial electron-transport chain, ascorbate-phenazine methosulphate-ferricyanide, was used to drive ATP synthesis in vesicles containing purified yeast F_1-F_0 ATPase (Ryrie and Blackmore, 1976). Such simplified systems appear to mimic the energization of ATP synthesis *in vivo*.

(d) The role of protons in ATP synthesis and hydrolysis

In the ATP synthesis direction of equation (1), two protons could arise from the de-protonation of both $ADPOH^{2-}$ and HPO_4^{2-}, balancing the $2H^+$ consumed during ATP^{4-} synthesis. The reverse situation applies to ATP-hydrolysis. The question of whether protons which may be involved in such a reaction are identical with those translocated during the reaction, remains unanswered, and is a principle point of difference between some of the models which are currently proposed.

G. CURRENT PROPOSALS FOR THE MECHANISMS OF ATP SYNTHESIS AND HYDROLYSIS BY THE MEMBRANE Mg-ATPase

(a) The proposal of Mitchell

Mitchell (1977) has elaborated on an earlier proposal (Mitchell, 1976) for the mechanism of ATP synthesis. A representation of the latest scheme is shown in Fig.I.1a, depicted as for bacteria Several points of interest should be noted:

(i) the overall reaction is

$$ATP^{4-} + H_2O + 2H^+_{in} \rightleftharpoons ADPOH^{2-} + HPO_4^{2-} + 2H^+_{out} \qquad \text{Equation (2)}$$

and is in effect a net translocation of $2H^+$ across the membrane; 'in' for synthesis, and 'out' for hydrolysis;

(ii) during ATP synthesis, the protons lost from the outer aqueous phase are incorporated into water;

(iii) the mechanism as written requires that $ADPOH^{2-}$ and HPO_4^{2-} (not ADP^{3-} and PO_4^{3-}), and ATP^{4-}, be the species that are present in the cytoplasm;

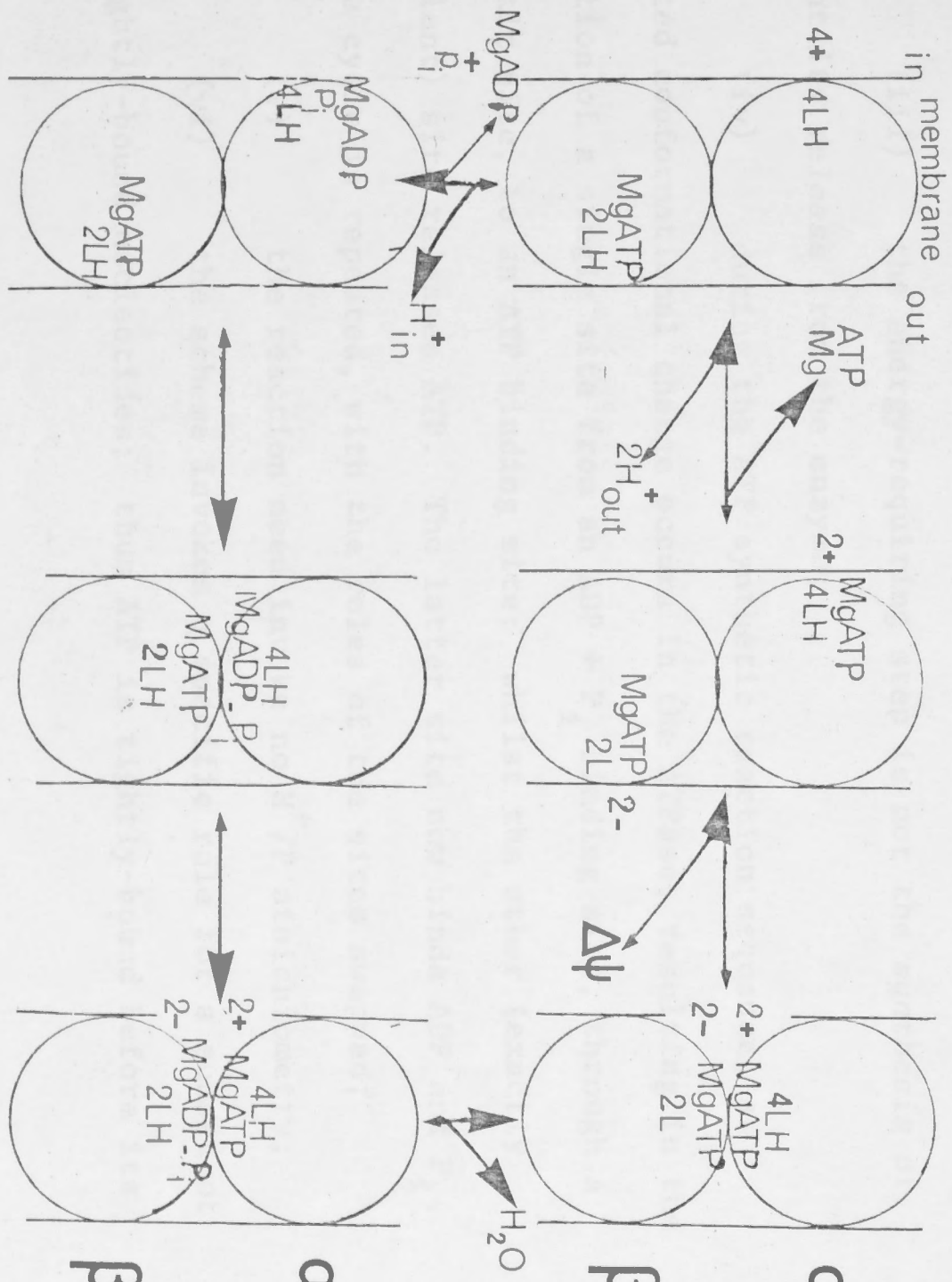
(iv) the reaction can be considered as a reversible O^{2-} group transfer between P_i and H_2O;

(v) the synthetic mechanism requires the attack of, from the outside, hydrated protons (averaging $H_3O^+ . 3H_2O$; Bell, 1959) and from the inside, ADP^{3-}, on the phosphate group;

(vi) the mechanism invokes three separate binding sites for the nucleotides, and two for the phosphate group. An 'antiport' is envisaged during ATP synthesis to remove ATP from the catalytic site to a separate site whilst moving ADP and P_i from the site of their initial binding (and de-protonation) to the catalytic site. The reverse

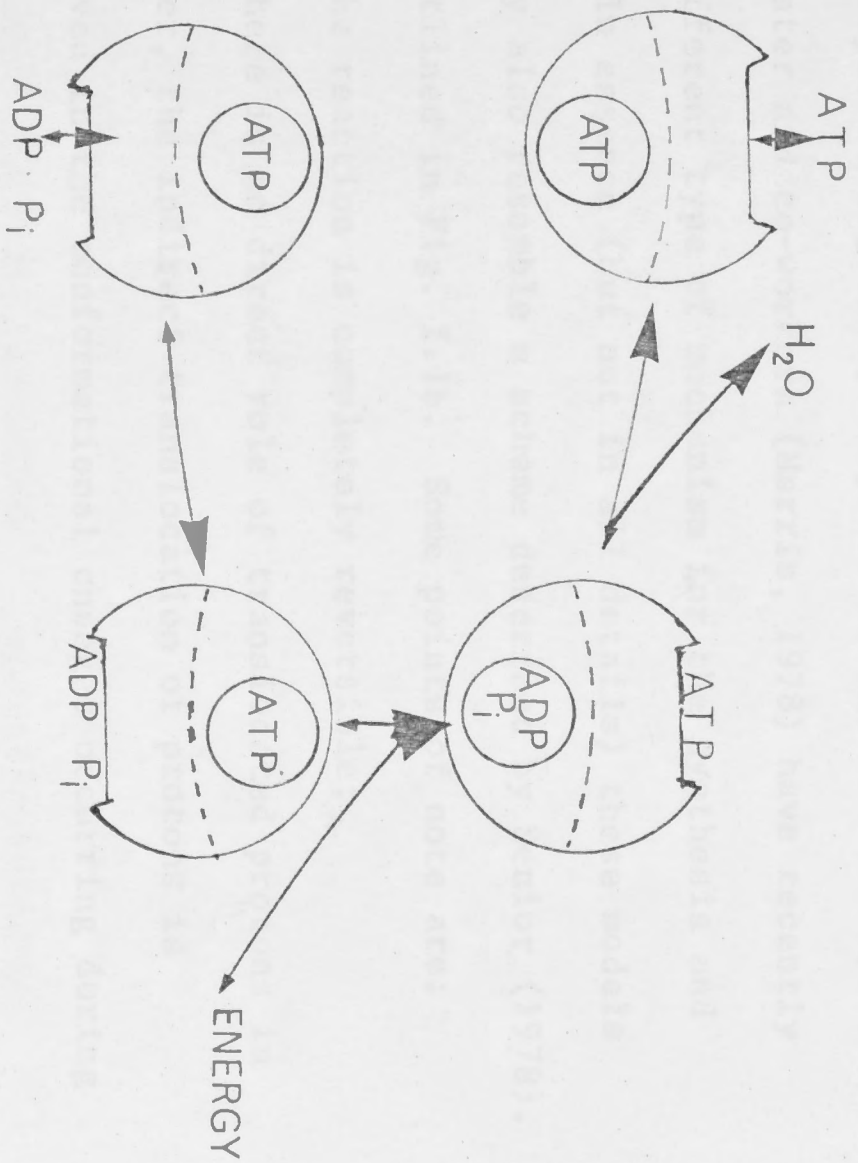
(C) THE MODEL OF KOZLOV AND SKULACHEV

L = $-NH_2$ group of lysyl residue
LH = L protonated



(B) THE MODEL OF BOYER

○ = 'tightly-bound' nucleotide
⌣ = equilibrium binding site



(A) THE MODEL OF MITCHELL

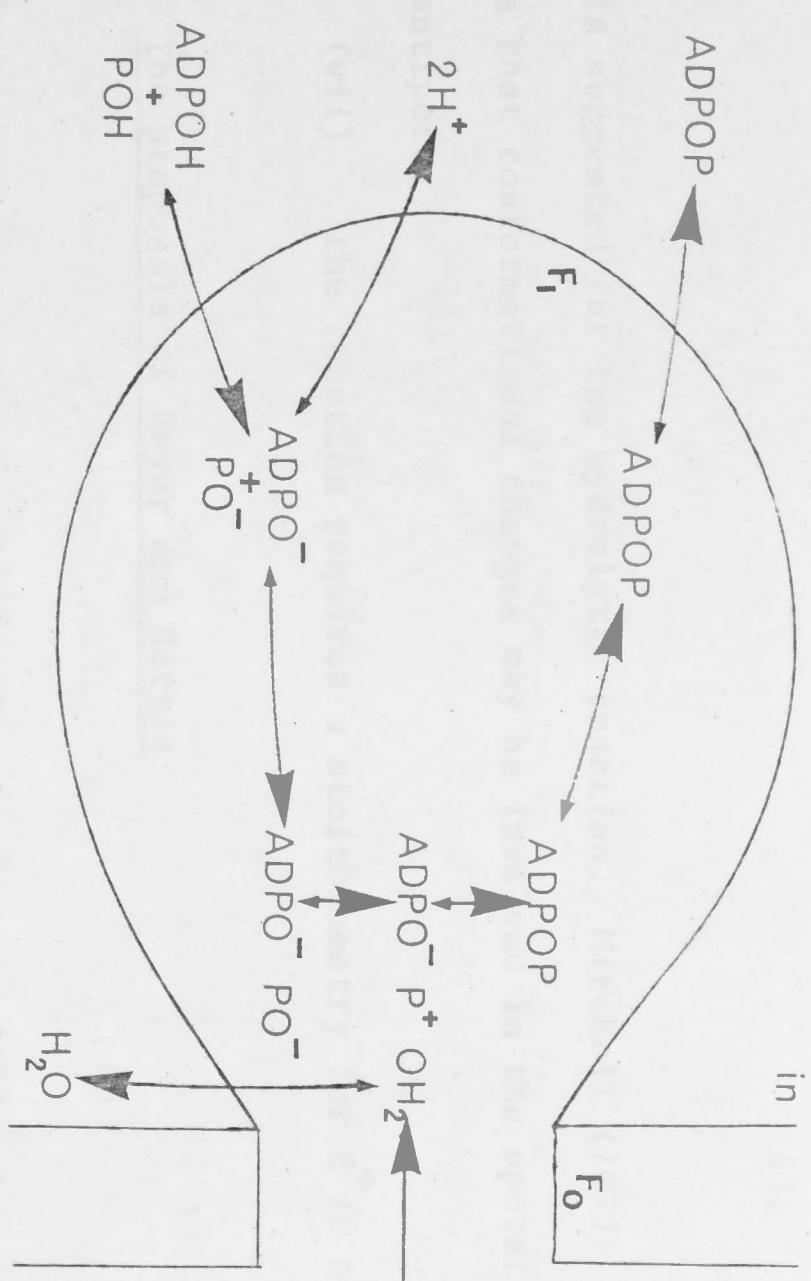


FIGURE I.1

process is suggested for the hydrolytic reaction. Mitchell (1977) considers that conformational changes may be involved in the operation of this antiport;

(vii) the reaction requires a stoichiometry for H^+/P of 2.

(b) The proposals of Boyer and Harris

Boyer (*in* Boyer *et al*, 1977; Kayalar *et al*, 1977; Boyer, 1975) and Harris, Slater and co-workers (Harris, 1978) have recently proposed a rather different type of mechanism for the synthesis and hydrolysis of ATP. In essence (but not in all details) these models are similar, and they also resemble a scheme described by Senior (1978). The proposals are outlined in Fig. I.1b. Some points of note are:

(i) the reaction is completely reversible;

(ii) there is no direct role of translocated protons in ATP synthesis; rather, the indirect translocation of protons is presumed to be involved in the conformational changes occurring during the reaction;

(iii) the energy-requiring step is not the *synthesis* of ATP, but its *release* from the enzyme;

(iv) during the ATP synthetic reaction sequence, a concerted conformational change occurs in the ATPase, resulting in the transition of a single site from an ADP + P_i binding site, through a catalytic site, to an ATP binding site; whilst the other (exactly equivalent) site releases ATP. The latter site now binds ADP and P_i, and the cycle is repeated, with the roles of the sites swapped;

(v) the reaction need invoke no H^+/P stoichiometry;

(vi) the scheme invokes a specific role for a subset of the tightly-bound nucleotides; thus ATP is tightly-bound before its

energy-dependent release, and ADP is tightly-bound after the conformational change leading to ATP synthesis (see especially Senior, 1978, and Harris, 1978);

(vii) in the model of Harris (1978), the release of ADP + P_i following ATPase activity involves a small input of energy.

(viii) in the model of Harris (1978), uncoupled ATPase activity occurs without a conformational change, requiring only one of the two sites, and does not involve a tightly-bound intermediate species. Thus ATP hydrolysis by a solubilized ATPase is seen as a completely separate reaction to the energy-generating ATP hydrolysis by a coupled ATPase.

(c) The proposal of Kozlov and Skulachev

Yet another speculative model is that proposed by Kozlov and Skulachev (1977), and shown in its simplest form in Fig.I.1c. The proposal is developed in great detail in Kozlov and Skulachev (1977). Even in the form shown in Fig.I.1c, detailed and specific roles for lysyl residues, Mg^{2+} ions, and the α- and β-subunits are invoked. Some features of this model are:

(i) the reaction is completely reversible;

(ii) the energy requirement (like that in the Boyer/Harris scheme) is for a conformational change leading to the release of tightly-bound ATP;

(iii) the model invokes a catalytic nucleotide-binding site (on the β-subunit) and a non-catalytic one (on the α-subunit);

(iv) protons are pumped between the aqueous phases in the same directions as hypothesized by Mitchell. However, the protons are not dehydrated directly during ATP synthesis; rather they are

first dehydrated by binding to lysyl-NH_2 groups (in the catalytic site in which $Mg.ATP^{2-}$ is bound), then the lysyl-NH_3^+ groups serve to dehydrate $Mg.ADP + P_i$ in the catalytic site. Net proton translocation occurs, since the binding of the substrates ($ADP + P_i$) to the non-catalytic site releases one proton to the medium;

(v) the model depends heavily on the net charge of bound substrates and products. Thus, $Mg.ADP^- + POH^{2-}$ bind to 4 lysyl-NH_3^+ groups in the α-subunit (non-catalytic site), expelling one H^+. The resulting complex ($ADP.Mg.PO_4.4NH_3$) is uncharged, and is envisaged to move into the hydrophobic sector, where the $(ADP.Mg.PO_4)^{4-}$ swaps with $(ATP.Mg)^{2-}$ bound in the catalytic site. This process results in, at the catalytic site, $(ADP.Mg.PO_4.2NH_3)^{2-}$, and at the non-catalytic site $(ATP.Mg.4NH_3)^{2+}$. In the catalytic site, two NH_3^+ groups promote the dehydration reaction. Energy is now required to separate the charged regions containing nucleotide, resulting in the release of ATP bound at the non-catalytic site;

(vi) the model employs the $\Delta\psi$ as the means by which these charges can be separated, and the ΔpH as the driving force for protonating, from the outside, the catalytic site -NH_2 groups;

(vii) the "tightly-bound" ATP is envisaged as that formed by the dehydration reaction, which is not released to the medium until the end of the next cycle of ATP synthesis. The catalytic site (in the β-subunit) is not directly accessible to nucleotides.

(d) The proposal of Williams

Williams (1978) has re-stated his earlier proposals that the protons involved in ATP synthesis are not in equilibrium with the aqueous phase. The protons generated by electron-transport are

envisaged as being trapped within a channel of immobilized water molecules, such that the proton activity is high, and protons are relatively dehydrated. Such an environment is envisaged to promote the dehydration of ADP + P_i in the active site. The model does not require a topologically-closed membrane, and the spatial organization of the model has been developed only sparingly by Williams (1978).

(e) The proposal of Robertson

The proposal of Robertson and Boardman (1975) invokes anhydrous HCl, trapped within the membrane, as the dehydrating agent for ATP synthesis. A novel, speculative, but not yet well-supported mechanism has been outlined in some detail in Robertson and Boardman (1975), Robertson and Thompson (1977) and Robertson (1978). In essence, Cl^- ions (or other anions) are trapped within the membrane, stored in a dehydrated state by the Fe^{3+} centres of cytochromes or non-haem iron. Oxidation results in the translocation of protons from the inner aqueous phase (in the case of bacteria) to the interior of the membrane, mediated by ubiquinone. The Cl^- ions act as H^+ acceptors, and the resulting HCl molecules are anhydrous, covalent, and quite soluble in the lipid environment. They are mobile within the membrane, and presumably interact with the proton channel in the F_0-sector of the Mg-ATPase.

The proposals of Robertson and of Williams, in which the protons are trapped within the membrane, are not-obviously compatible with the evidence, chiefly from *in vitro* experiments, that proton translocation can be measured in the aqueous phases. Williams (1978) maintains that the immobilized water channel can be in contact with

the aqueous phase, but that the exchange of molecules is very slow compared with the reactions occurring within the hydrophobic phase, and so the energy "stored" in the relatively-dehydrated proton is conserved. Robertson and Boardman (1975) have invoked the structure of the tightly-packed phospholipid polar headgroups, stabilized by divalent cations, for the role of the containment of the HCl molecules. The ionic environment of the polar headgroups, as noted before, controls not only the packing, but also the fluidity of the phospholipids (Träuble and Eibl, 1974), and these properties would therefore be expected to be somewhat different on the opposite surfaces of the membrane, especially in *in vitro* experiments. The transport of HCl to the aqueous phase could be mediated by the polar headgroup of phosphatidylcholine, accessible to anhydrous HCl only when the packing of the phospholipids near the polar region was loose, and the fluidity high (see also Robertson and Thompson, 1977, and Robertson, 1978). During the application of an artificial proton-motive force, under conditions where the KCl concentration is high and the divalent cation concentration low (e.g. Tsuchiya, 1977), HCl could similarly penetrate into the membrane via phosphatidylcholine.

An important aspect of the hypotheses involving 'dry' protons, is that the ATP synthesis reaction does not require a large energy input for the dehydration of two protons, as it does for instance in the mechanism of Mitchell (1976, 1977). However, in the hypothesis of Robertson and Boardman (1975), the H-Cl covalent bond would have to be cleaved.

An interesting sidelight to this proposal is the recent identification of an iron-sulphur flavoprotein in a purified F_1-F_0 ATPase from beef heart mitochondria (Stigall *et al*, 1978). The

lipophilic iron chelator bathophenanthroline is known to inhibit membrane bound Mg-ATPase activity in *E.coli* (Sun *et al*, 1975), presumably by interacting with an analogous protein. The membrane-bound Mg-ATPase of an *uncC424* mutant, but not an *uncB402* mutant, was also sensitive to the chelator (Cox *et al*, 1977; Sun *et al*, 1975). The potential role of such a protein is obscure; perhaps the iron centres are able to store Cl^- ions, as would be required by the Robertson scheme.

(f) A comparison of some aspects of the various proposals

At the outset, it should be made clear that any or none of these proposals may represent the true reaction. Such models are nevertheless useful, in that they have implications which may be tested experimentally. Some general attributes and deficiencies of the various models are now discussed.

(i) Proton movements

Williams (1978) has pointed out that the containment of a relatively-anhydrous proton within the membrane is a more economical and controllable way of conserving its energy, than the release of the proton into the aqueous medium, where spontaneous hydration and concomitant loss of potential energy would occur. The 'localized proton' hypotheses also allow for the discriminatory or selective coupling of energy to different processes. This idea is compatible with the 'energy-transfer domain' concept (Ernster *in* Boyer *et al*, 1977) in which several complexes carrying out a specific process are clustered together in the membrane, interacting rather more directly than via an

electrochemical gradient across the membrane. In the hypothesis of Robertson and Boardman (1975), the cluster may be defined by the extent of a linked patch of phospholipid bilayer, allowing 'localized' energy transfer (by diffusion of HCl) over large distances, as is required in chloroplasts, where the site of energy generation in the grana stacks is a large distance from the ATP-synthesizing complexes in the unstacked regions (Miller and Staehelin, 1976). The latter situation is difficult to explain in terms of the hypothesis of Williams (1978), but is compatible with each of the other hypotheses.

The idea that proton efflux into the aqueous layer might not be a primary event is supported by the observation of slow rates of acidification of the outer medium after the addition of oxygen to anaerobic or energy-depleted cells of *E.coli* (Gould and Cramer, 1977; cf. Wilson *et al*, 1976). The question of the buffering capacity of the inner and outer phases is still a matter for debate (cf. Mitchell, 1977; Williams, 1978). As stressed by Mitchell (1977), however, the translocation of a proton across the membrane also involves an electrical component. Witt *et al* (1976) observed that ATP synthesis could occur in response to a membrane potential only. The model of Mitchell (1977) requires that proton translocation occurs in such a situation.

Even if the proton influx and efflux were not primary events, the involvement of proton symports in transport is well-established (Simoni and Postma, 1975; Hamilton, 1977; Harold, 1977a). The detection of localized ion gradients around a cell or organism (see Harold, 1977b) poses some intriguing questions in relation to the localization of certain energetic processes, such as ATP synthesis, active transport, and chemotaxis.

Yet another possibility has been raised by Archbold and co-workers (see Archbold *et al*, 1976), who interpreted their results in terms of a proton-motive force (principally electrostatic) involving a zone of fixed charges on the surfaces of the membrane. These workers also pointed out that such a mode of energy conservation is compatible with the 'energy-transfer domain' concept mentioned above, whereas the chemiosmotic hypothesis is not. Ort and Dilley (1976) and Ort *et al* (1976) have also provided evidence for localized proton-conducting paths close to the membrane surface.

Another question relevant to the generation of a trans-membrane pH gradient was raised by Garland (1977) and Garland and Haddock (1977). These workers pointed out that the mechanism of membrane energization proposed by the chemiosmotic hypothesis runs into difficulties in the case of alkalophilic bacteria, living in an outer environment of pH 9. The proposals of Robertson, Williams or Archbold could more easily explain the energization of such membranes. The data of Ramos *et al* (1976) would suggest that the ΔpH is insignificant at alkaline pH in membrane vesicles of *E.coli*, and moreover that the proton-motive force is considerably lower at alkaline pH than at acidic pH. It is possible that intramembrane energization and a transmembrane proton-motive potential difference could be both operative in biological membranes.

The role of the F_0-sector of the Mg-ATPase as a proton channel is compatible with each model. However, the nature of the proton channel is rather different in each of the five models discussed. In the model of Mitchell, the channel is an aqueous well leading to the catalytic site. The proposal of Williams invokes a channel of

immobilized water, whilst that in the type of scheme proposed by Robertson might be a non-aqueous channel (see also Nagle and Morowitz, 1978). The proton channel in the Boyer model is an indirect one, whilst the proton channel in the Kozlov/Skulachev scheme gives access to the lysyl groups of the catalytic site.

The question of H^+/P stoichiometry is a point at issue. The model of Boyer requires no stoichiometry. In refuting the contrary assertions of Mitchell (1977), Williams (1978) states that no stoichiometry can be invoked for his hypothesis. The proposal of Mitchell (1977) on the other hand, invokes a H^+/P stoichiometry of 2 in the directions of ATP synthesis or hydrolysis. Measurement of this ratio is altogether another problem, as severe complications arise from back-diffusion of protons with anions such as formate and possibly phosphate, and in mitochondria, from the additional complexity of the electrogenic translocation of adenine nucleotides (see Garland and Haddock, 1977; Garland, 1977). Moreover, if the species of ADP and ATP involved were ADP^{3-} and ATP^{4-}, and not $ADPOH^{2-}$ and ATP^{4-} as required by Equation (2), an H^+/P stoichiometry of 2 would not be reflected in the proton movements measured in the bulk phase. The model of Kozlov and Skulachev (1977) in effect recognizes this point.

(ii) Conformational changes

A notable similarity between several of the models outlined above, is the incorporation of a concerted conformational change into the mechanism. In the schemes of Mitchell and of Kozlov and Skulachev, the conformational change is associated with an antiport, or simultaneous translocation of ATP from the catalytic site, and

translocation of ADP + P_i to the catalytic site. Two non-catalytic sites are involved in the model of Mitchell (1977), and only one in the model of Kozlov and Skulachev (1977). The latter workers propose a swapping mechanism involving rotation of a pentavalent phosphorus intermediate. The conformational changes in the scheme of Kayalar *et al* (1977) do not involve the translocation of nucleotides between sites. In this model, conformational changes are considered to link proton movement with the concerted alterations in the character of pairs of nucleotide-binding sites (Boyer, 1975; *in* Boyer *et al*, 1977; Rosing *et al*, 1977; Kayalar *et al*, 1977). There is considerable evidence for some kind of conformational change (see section E(c) above).

(iii) Tightly-bound nucleotides

Maeda *et al* (1977b) have found that there is ATP bound to the membrane Mg-ATPase of *E.coli*, which remains tightly-bound and non-exchangeable under energized conditions, and does not participate in the reaction directly. Such ATP may be bound in regulatory sites (see section E(b) above). There are other tightly-bound nucleotides under de-energized conditions; these are involved in the reversible ATPase reaction in the models of Harris (1978), Senior (1978), Boyer (*in* Boyer *et al*, 1977; Kayalar *et al*, 1977), and Kozlov and Skulachev (1977). The model of Mitchell (1977) could easily be adapted to include a role for such tightly-bound nucleotides.

(iv) Uncouplers

Uncouplers are molecules which uncouple respiration from ATP synthesis and other energy-requiring reactions, by dispersing the

energized state of the membrane. In terms of hypothesis involving protonic potential difference in some form, uncouplers would act as a proton ionophore. There is, however, disagreement as to the mode of action of uncouplers, and it appears that it is not *necessary* to collapse the protonic potential difference for uncoupling (Hanstein and Hatefi, 1974; Hatefi, 1975). The role of uncouplers is not yet defined well-enough to draw any conclusions about mechanistic models for Mg-ATPase activity.

(v) Exchange reactions

Several exchange reactions can be measured in the energized membrane-bound Mg-ATPase, including $ATP \rightleftharpoons {}^{32}P_i$, $ATP \rightleftharpoons H^{18}OH$, and $P_i \rightleftharpoons H^{18}OH$. These reactions are considered to reflect steps in the reversible ATPase reaction. There is some evidence that only one exchange reaction occurs under de-energized conditions (e.g. in the presence of uncouplers): bound $P_i \rightleftharpoons H^{18}OH$ (Rosing *et al*, 1977; Russo *et al*, 1978). The free $P_i \rightleftharpoons HOH$ exchange, on the other hand requires energy, and is sensitive to uncouplers (Rosing *et al*, 1977). The models of Boyer, Harris and Kozlov and Skulachev were formulated in terms of experimentally-established features of the exchange reactions, in particular, the observation that the $P_i \rightleftharpoons H^{18}OH$ and $ATP \rightleftharpoons H^{18}OH$ exchange reactions are more rapid than the ${}^{32}P_i \rightleftharpoons ATP$ exchange.

The observation that a soluble F_1-F_0 ATPase preparation (Complex V) could carry out a rapid $ATP \rightleftharpoons {}^{33}P_i$ exchange reaction in the apparent absence of a topologically-closed membrane structure (Stiggall *et al*, 1978), may cast some doubt on the dependence on energy and the reversible proton-pumping postulated for this exchange. However, it is

also possible that Complex V is energized; for instance, by allowing proton movements in a phase of immobilized water as per the Williams model. It is noteworthy that the presence of phospholipids is obligatory for the reaction to occur; according to the Robertson scheme, the phospholipids would provide a reservoir for HCl. Schairer *et al* (1976) found that they could correlate the presence or absence of the $ATP \rightleftharpoons {}^{32}P_i$ exchange in *E.coli* membranes with the presence or absence of oxidative phosphorylation, the Mg-ATPase, or ATP-dependent activities. In the *unc* ATPase$^+$ strains DG15/10, DG26/4, DG7/10, and DG31/3 (Table I.2), the $ATP \rightleftharpoons {}^{32}P_i$ exchange was lacking, suggesting a requirement for a coupled Mg-ATPase (Schairer *et al*, 1976).

It should be emphasized that the $ATP \rightleftharpoons P_i$ exchange involves the continual binding to and dissociation from the Mg-ATPase of both ATP and P_i. The bound $P_i \rightleftharpoons H_2O$ exchange involves only the 'binding' of H_2O. It could be that a more rigid conformational structure is required for the former exchange reaction than for the latter.

(vi) Other aspects of the proposals

Studies using nucleotide analogues and various specific inhibitors have not so far eliminated any of the proposals discussed. However, there are indications that these compounds may be very useful in probing the mechanisms of ATP synthesis and hydrolysis (see e.g. Senior, 1978; and Kozlov and Skulachev, 1977).

(g) The possible role of lipoic acid and unsaturated fatty acids

Griffiths and co-workers have recently demonstrated an

apparent net synthesis of ATP dependent on dihydrolipoic acid and unsaturated fatty acids such as oleic acid. Such synthesis occurred in *E.coli* membranes (Partis *et al*, 1977), and yeast (Griffiths *et al*, 1977a, b, c; Griffiths and Hyams, 1977) and beef heart (Griffiths, 1976) mitochondria. Lipoic acid also appeared to be necessary for light-dependent ATP synthesis in membrane vesicles from *E.coli* containing bacteriorhodopsin, or in vesicles containing purified mitochondrial F_1-F_0 ATPase and bacteriorhodopsin (Griffiths *et al*, 1977d). Haddock and Begg (1977) subsequently showed that lipoic acid was not required for the ATP-driven energization of *E.coli* membranes, as measured by atebrin fluorescence quenching. Further investigations into the nature of the dihydrolipoate and oleic acid requirements are therefore necessary.

H. EXPERIMENTAL INVESTIGATIONS AIMED AT THE EVENTUAL ELUCIDATION OF THE STRUCTURE AND FUNCTION OF THE Mg-ATPase

As noted in section A of this chapter, the use of *unc* mutant strains of *E.coli* promised to be a powerful tool for studying the biosynthesis of the Mg-ATPase, the structural and functional roles of its components, and the catalytic mechanism. The studies presented in this thesis were undertaken with the general aim of clarifying the protein structure of certain *unc* mutants, to provide a basis for investigations into the structural and mechanistic aspects of the Mg-ATPase.

Chapter II

MATERIALS AND METHODS

A. MATERIALS

Chemicals were of the highest purity available commercially and were not further purified. Acrylamide, N,N'-methylenebisacrylamide, and N,N,N',N'-tetramethylethylenediamine (Temed) were from Eastman Kodak, Rochester, N.Y., U.S.A. Sodium dodecyl sulphate (SDS) (especially pure) and Nonidet P40 were from B.D.H., Poole, England. Coomassie Blue R250 was from Serva, Heidelberg, Germany. Atebrin (quinacrine hydrochloride), p-Aminobenzamidine hydrochloride, urea, ATP and NADH were from Sigma Chemical Co., St. Louis, Mo., U.S.A. N,N'-dicyclohexyl-carbodiimide and ε-amino-n-caproic acid were from Fluka A.G., Buchs, Switzerland. Ampholines were from LKB Produkter AB, Bromma, Sweden. Streptomycin was from Glaxo, Boronia, Victoria, Australia. Nalidixic acid was from Calbiochem, La Jolla, Cal., U.S.A. $^{32}P_i$ (carrier-free) was obtained from the Australian Atomic Energy Commission, Lucas Heights, N.S.W., Australia. Phosphorylase A and β-galactosidase were from Worthington Biochemical Corporation, Freehold, N.J., U.S.A. Bovine serum albumin (Fraction V), ovalbumin, horse-spleen ferritin, catalase, rabbit-muscle adenylate kinase and ribonuclease A were from Sigma. Bovine liver glutamate dehydrogenase and rabbit muscle triosephosphate isomerase were from Boehringer, Mannheim, Germany. Horse liver alcohol dehydrogenase and horse heart cytochrome c were from Calbiochem. Zinc-free bovine insulin was kindly given by Dr. B. K. Milthorpe, and jack-bean urease by Dr. J. N. Burnell.

B. MEDIA

(a) Minimal salts medium

The minimal salts medium used for the growth of bacteria was similar to the 'Medium 56' of Monod *et al* (1951), supplemented with trace metals.

20 X '56'		
	K_2HPO_4	212g
	$NaH_2PO_4 2H_2O$	122g
	$(NH_4)_2SO_4$	40g
	H_2O	1 litre

3 ml of toluene and 3 ml of chloroform were mixed and added and the solution was stored at room temperature. 50 ml of 20 x '56' was used per litre of growth medium.

A concentrated trace-element suspension was added to the diluted salts medium. The composition of the trace metal supplement (Gibson *et al*, 1977b) is shown below. The weight of each salt added per litre of water is listed, followed by the concentration of the salt in the growth medium (in brackets):

$FeCl_3.6H_2O$	0.48g	(1.78μM)
$CaCl_2.2H_2O$	0.36g	(2.45μM)
$MnSO_4.H_2O$	0.17g	(1μM)
$ZnSO_4.7H_2O$	4.0g	(13.9μM)
$CoSO_4.7H_2O$	0.19g	(0.68μM)
H_3BO_3	0.29g	(4.69μM)

After sterilization of the diluted salts medium, 1 ml of a sterile

1M $MgSO_4$ solution was added per litre of growth medium.

(b) Luria broth

This medium was described by Luria and Burrous (1957) and consisted of:

Bacto-Tryptone (Difco)	10g
Yeast Extract (Difco)	5g
NaCl	10g
H_2O	1 litre

The pH of the broth was adjusted to 7.0 with 10N NaOH before autoclaving.

(c) Growth supplements

All growth supplements were sterilized separately and added to the sterilized salts medium. Either glucose or succinate was used as the carbon source, at a final concentration of 33 mM. Other growth supplements were used when necessary at the following final concentrations:
Luria broth, 5% (v/v); thiamine hydrochloride, 1.5μM; L-arginine hydrochloride, 0.8mM; 2,3-dihydroxybenzoate, 30μM; uracil, 0.2mM; adenine, 0.15mM; casein hydrolysate (casamino acids), 0.05%.

(d) Z broth

Z broth was used for the growth of bacteriophage P1*kc* and for transduction experiments, and consisted of Luria broth supplemented with 2.5mM-$CaCl_2$. Glucose (33mM) was added where

necessary.

(e) Glycerol-Luria broth

This broth was used for the storage of strains at -18°C, and was prepared by adding 70ml of Luria broth to 30ml of sterilized glycerol.

(f) Nutrient agar (Brain Heart Infusion agar - BIA)

Brain Heart Infusion	37g
Agar (Difco)	20g
H_2O	1 litre

This mixture was dissolved by autoclaving, and 57mM glucose was added when required.

(g) Nutrient agar (Meat Infusion agar - MIA)

Peptone (Oxoid)	10g
Beef extract (Difco)	10g
Yeast extract (Difco)	3g
NaCl	5g
H_2O	1 litre

The mixture was dissolved, and the pH adjusted to 7.5. 20g Difco agar was included before autoclaving. Glucose (final concentration 30mM) was added when required.

(h) Solid media

Soft agars, used for growth of phage P1*kc*, consisted of 0.8% Difco Bacto-agar in diluted salts medium (including $MgSO_4$). Z Plates contained 1% agar in Z broth. All other solid media were prepared by adding 2% (w/v) Difco Bacto-agar to the appropriate liquid medium.

C. ORGANISMS

All the bacterial strains used were derived from *E.coli* K-12 and are described in Table II.1. Bacteria were stored as frozen ($-18^{o}C$) suspensions in glycerol-Luria broth medium. The required strain was plated usually onto a nutrient agar plate, to give confluent growth, and the cells were resuspended in 3ml of glycerol-Luria. Partial diploid strains were always gown on minimal medium plates, to retain the plasmid.

The bacteriophage P1*kc* was used in transduction experiments.

D. GROWTH MEASUREMENTS IN LIQUID MEDIA

Strains were grown in 125ml conical side-arm flasks containing 10ml of medium. Turbidity was measured in a Klett-Summerson colorimeter with a blue filter, against a blank of the same medium. Cell density of the culture was expressed in arbitrary Klett units. For the measurement of growth yield on limiting

Table II.1 *Strains of E.coli K12 used*

The gene designations follow those of Bachmann *et al.* (1976), and the plasmid nomenclature is that of Novick *et al.* (1976).

Bacterial strain	Relevant genetic loci	Other information
AB259	Hfr	Hfr Hayes
AN248	F^-, *ilvC7, argH1, entA*	Butlin *et al* (1973)
AN249	F^-, *uncA401, argH1, entA*	Cox *et al* (1973b)
AN283	F^-, *uncB402, argH1, entA*	Butlin *et al* (1973)
AN285	F^-, *uncD405, argH1, entA*	Cox *et al* (1974)
AN295	F^-, *ilvC7, entA* (de-repressed Mg-ATPase)	Cox *et al* (1973a)
AN346	F^-, *ilvC7, argH1, pyrE, entA*	Gibson *et al* (1977a)
AN463	F', *uncD409, argH1, entA*	Isolated after transduction with strain *M54* (Cox *et al*, 1978) as donor and strain AN248 as recipient
AN716	F^-, *uncB402, argH1, entA, hemA, leu*	Cox *et al* (1977)
AN1007	F^-, *unc-436, argH1, entA, pyrE,* Str^R	Isolated after transduction with mutated P1 grown on strain AB259, with strain AN346 as the recipient.
AN1008	F^-, *unc-436, argH1, entA, pyrE,* Str^R, *recA, nalA*	Isolated after mating between strains KL163 and AN1007.
AN1064	F^-, *unc-441, argH1, entA, pyrE,* Str^R	Isolated after transduction with mutated P1 grown on strain AB259, with strain AN346 as the recipient.
AN1065	F^-, *unc-441, argH1, entA, pyrE,* Str^R, *recA, nalA*	Isolated after mating between strains KL163 and AN1064.
KL163	Hfr, *nalA, recA*	Obtained from J. Pittard.
W3110	$trpE9829_{am}$	Obtained from C. Yanofsky.
Diploids		
AN785	F' (pAN2), *uncD405, argH1, pyrE, entA,* Str^R, *recA, nalA*	Isolated after mating between strains AN780 (Cox *et al*, 1978) and AN728 (Gibson *et al*, 1977a).
AN821	F' (pAN7), *ilvC7, argH1, pyrE, entA,* Str^R, *recA, nalA*	Cox *et al* (1978).
AN834	F' (pAN7), *uncD405, argH1, pyrE, entA,* Str^R, *recA, nalA*	Isolated after mating between strains AN819 (Cox *et al*, 1978) and AN728 (Gibson *et al*, 1977a).
Plasmids		
pAN2	*uncA401,* $ilvC^+$, $argH^+$, $pyrE^+$	Cox *et al*, 1978.
pAN7	*uncD409,* $ilvC^+$, $argH^+$, $pyrE^+$	Cox *et al*, 1978.
pAN11	unc^+, $ilvC^+$, $argH^+$, $pyrE^+$	Cox *et al*, 1978.

glucose (Cox *et al*, 1970), minimal medium containing 5mM glucose was used.

E. GENETIC TECHNIQUES

(a) Preparation of phage lysates for generalized transduction

The donor strain was grown to stationary phase in 10ml of Z broth, or 10ml of glucose Z broth for strains carrying *unc*$^-$ mutations. The cell suspension was centrifuged and resuspended in 1ml of fresh Z broth. 1ml of a preparation of the generalized transducing phage P1*kc* (containing about 10^9 p.f.u.) was added, and samples (0.2ml) of the mixture were transferred to 3ml molten soft agars held at 45^{o}C. After gentle mixing, the soft agars were poured as overlays on Z plates. The plates were incubated at 37^{o}C overnight, then the overlay was resuspended in 2ml of Z broth and centrifuged at 25,000g for 20 min to remove agar and bacterial debris. The supernatant containing the phage particles was sterilized by gentle mixing with about 0.05 vol of a suspension of chloroform in Z broth. The phage lysate was stored at 4^{o}C, and only the clear aqueous layer was used for transduction experiments.

(b) Transduction procedure

The technique used for transduction experiments was described by Pittard (1965). A culture of recipient bacteria was grown overnight in 10ml of Z broth, centrifuged, and resuspended in 1ml of Z broth. 0.1ml of this cell suspension, containing about 10^9

cells, was added to 2ml of fresh Z broth, and then 0.5ml of phage lysate from the donor strain, containing approximately 10^9 p.f.u., was added. The mixture was incubated without agitation at 37°C for 20 min to allow phage adsorption. The mixture was then centrifuged and the pellet resuspended in 1ml of the same medium. Samples (0.1ml) of the undiluted cell suspension and of a 1 in 10 dilution, were spread on appropriate selective media and incubated at 37°C. Transductants appeared after about two days.

(c) Conjugation technique for mating experiments

The technique used for mating experiments was based on that described by Taylor and Thoman (1964). The male and female strains were grown on MIA plates overnight. Small inocula were added to 10ml of Luria broth in side-arm flasks, and each strain grown to give a Klett reading of about 70. Glucose was included in solid and liquid media when uncoupled strains or recombination-deficient strains were grown. At zero time, 1ml of the male culture was then mixed with 9ml of the female culture in a side-arm flask. The mixture was incubated at 37°C without agitation, and 1ml samples were taken at appropriate times. Each sample was agitated vigorously for 45 sec using a vortex mixer, diluted and screened for bacteria carrying the required transferred markers.

Recombination-deficient (*recA*) derivatives of female strains were obtained using this method (Gibson *et al*, 1977a). The Hfr male strain KL163 (*recA*, *nalA*) was used as the donor. Samples were taken at 20 min, 40 min and 50 min, diluted 10-fold with glucose-Luria containing 0.002% Streptomycin, and incubated at 37°C for 4 to 5 hours with shaking. Samples (0.1ml) of the undiluted cell

suspension and of a 1 in 10 dilution were spread onto BIA plates containing 0.02% Streptomycin and 0.1mM nalidixic acid. The male is sensitive to streptomycin, and the female to nalidixic acid. Only transconjugants containing *nalA* could grow, and these were screened for defective recombination, by streaking out single colonies and exposing part of each streak for 25 seconds to short-wave ultraviolet light. Recombination-deficient cells did not survive this treatment.

(d) Complementation tests

Strains of *E.coli* carrying a plasmid containing the region of the chromosome between *pyrE* and *argH* (which includes the *unc* and *ilv* genes) have been obtained (Gibson *et al*, 1977a). The construction of strains carrying various *unc* alleles on the plasmid, has allowed tests for complementation between different *unc* alleles (Gibson *et al*, 1977b). In this way, five distinct *unc* genes have been classified so far. Plasmids have been isolated containing the *uncA401* allele (Cox *et al*, 1978), the *uncB402* allele (Gibson *et al*, 1977b), the *uncC424* allele (Gibson *et al*, 1977b), the *uncD409* allele (Cox *et al*, 1978), the *uncE429* allele (J. A. Downie, unpublished work), or the unc^+ region (Cox *et al*, 1978; Gibson *et al*, 1977a).

The uncharacterized *unc* allele to be tested was introduced into a strain (AN346) containing *ilvC*, *argH* and *pyrE* mutations, by co-transduction with *ilv*. The ilv^+ transductants were screened for the presence of the *unc* phenotype. A *recA* derivative of one transductant was constructed and used as the recipient.

Male donors, carrying a *purE* allele on the chromosome, and the six plasmids listed above, were grown to a Klett reading of 70 in 10ml of glucose-Luria broth, centrifuged and resuspended in 1ml of diluted salts medium. The recipient strain was grown to a Klett reading of 60-100, and used undiluted. A loop of the recipient strain was streaked along each plate, and a loop of each donor strain was streaked across the plate at right angles. The selective media used contained succinate and casamino acids, but not adenine, uracil or arginine, in order to select against the donor and recipient, but allow adequate growth of transconjugants containing complementing *unc* alleles. Control plates containing uracil, arginine, succinate and casamino-acids allowed growth of revertants as well as the diploids containing complementing *unc* alleles. Plates containing glucose allowed growth only of transconjugants carrying complementing or non-complementing *unc* alleles, and were used for checking the transfer of the plasmid. Diploid strains were purified, and segregants obtained (Gibson *et al*, 1977a) after growth in Luria broth, plating on nutrient agar, and testing individual colonies on selective media.

F. 'LOCALIZED' MUTAGENESIS OF PHAGE P1

The procedure for mutagenesis of transducing phage, followed by selection of transductants containing mutations in specific regions of the chromosome, was based on those of Hong and Ames (1971) and Murgola and Yanofsky (1974). A lysate of phage P1 was prepared on an *ilv*$^{+}$ *unc*$^{+}$ strain, by the procedure described above, except that all volumes were increased 3-fold, and the

chloroform addition was omitted. The lysate was centrifuged at 37,000g for 30 min, and the pellet of phage was gently dislodged into 3ml of Z broth. The phage suspension was added to 27ml of 0.45M-hydroxylamine hydrochloride, pH 6.0 with NaOH, containing 2mM-EDTA and 10mM-$CaCl_2$. The mixture was shaken gently at 37°C for 13.5 hours. The mutated phage were sedimented as before, and resuspended in 3ml of Luria broth containing 10mM-$CaCl_2$ and 2mM-EDTA. The phage preparation was sterilized with chloroform. This procedure gives mutated P1 particles, including a small number of transducing phage carrying mutations in the *unc* region of the chromosome.

The mutated phage was used to transduce the *ilvC* strain AN346 to *ilv*$^+$. The transductants were screened by replica-plating for inability to grow on succinate minimal medium.

G. GROWTH OF CELLS

Cells of the desired strain, grown confluently on a glucose minimal medium plate, were resuspended in dilute salts medium and added to 1 litre of glucose minimal medium in a 2 litre baffled flask. The flask was shaken overnight at 37°C and at 300 rev/min in a New Brunswick Gyrotory shaker. The inoculum was added to 10 litres of glucose minimal medium in a 14-litre New Brunswick fermenter which was kept at 37°C, aerated at 12 litre/min and stirred at 400 rev/min. Cells were grown to mid-exponential phase. At a Klett reading of about 240 (a cell density of about 0.5mg dry weight of cells/ml), the cells were harvested using a Sharples continuous centrifuge.

Harvested cells were resuspended in 0.1M-tris (hydroxymethyl)

methyl-2-aminoethanesulphonic acid (Tes) buffer, containing 0.25M-sucrose, 0.02M-magnesium acetate, 0.25M-EGTA, and 0.04M-ε-amino-n-caproic acid (EACA). This buffer system is referred to as STEM buffer. The cell suspension was centrifuged at 16,000g for 20 min. Washed cells were often stored at -80°C.

H. FRACTIONATION OF MEMBRANES AND SOLUBILIZATION OF Mg-ATPase

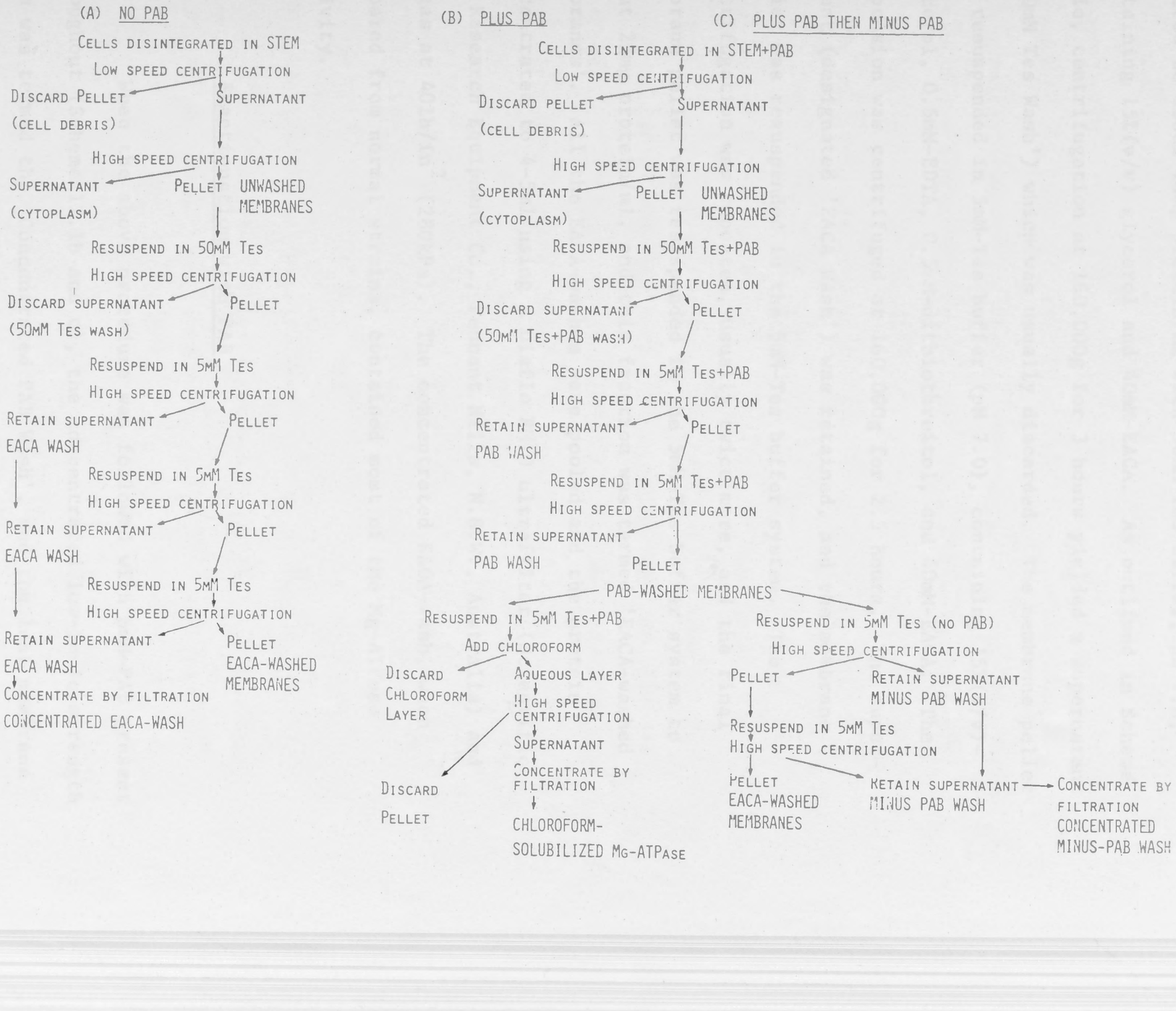
Membranes were prepared and fractionated as outlined in Scheme II.1. The fractionation procedure is based on the observation that the presence of the protease inhibitor p-aminobenzamidine (PAB) prevented the solubilization, by low-ionic strength washing, of membrane-bound Mg-ATPase activity.

Washed cells were resuspended in STEM buffer (see section G) without PAB (Scheme II.1a), or STEM buffer containing 6mM-PAB (Scheme II.1b and c), using 2ml of buffer per 1g (wet weight) of cells. The cells were then disintegrated by passage through a Sorvall Ribi Cell Fractionator at 20,000 lb/in^2 (137MPa). The cell extract was centrifuged at 27,000g for 30 min to remove cell debris. The supernatant was centrifuged in a Beckman L565 Ultracentrifuge at 160,000g for 3 hours, yielding a membrane pellet, and a supernatant containing cell cytoplasm. The membrane pellet was resuspended in fresh buffer to a protein concentration of 30-50mg/ml, and this preparation was termed 'Unwashed Membranes'.

(a) Fractionation without PAB

Unwashed membranes prepared in the absence of PAB were

SCHEME II.1. FLOW DIAGRAM FOR MEMBRANE FRACTIONATION AND SOLUBILIZATION OF Mg-ATPase



diluted to about 10mg protein/ml with 50mM-Tes buffer, pH 7.0, containing 15%(v/v) glycerol and 40mM-EACA. As outlined in Scheme II.1a, centrifugation at 160,000g for 3 hours yielded a supernatant ('50mM Tes Wash') which was usually discarded. The membrane pellet was resuspended in 5mM-Tes buffer (pH 7.0), containing 15%(v/v)-glycerol, 0.5mM-EDTA, 0.5mM-dithiothreitol, and 40mM-EACA. The suspension was centrifuged at 160,000g for 2.5 hours. The supernatant (designated 'EACA Wash') was retained, and the membrane pellet was resuspended in the 5mM-Tes buffer system. The centrifugation was repeated, usually twice more, and the final membrane pellet was resuspended in the 5mM-Tes buffer system to about 25mg protein/ml, and this fraction was termed 'EACA-washed membranes'. All the EACA-washes were pooled and the protein concentrated to 4-5ml using a Diaflo PM10 ultrafilter (Scientific and Research Equipment Co., Pennant Hills, N.S.W., Australia) and N_2 gas at 40lb/in^2 (280kPa). The concentrated EACA-wash, when prepared from normal strains, contained most of the Mg-ATPase activity.

(b) Fractionation with PAB

When the above procedure was followed with 6mM-PAB present throughout (Scheme II.1b and c), the concentrated low-ionic strength wash was termed the 'Concentrated PAB Wash', and the last membrane pellet, 'PAB-washed Membranes'. The third low-ionic strength wash was omitted. No Mg-ATPase activity was solubilized from membranes of a normal strain during this procedure. Two methods were used to solubilize the Mg-ATPase.

(i) Chloroform: water extraction method.

The chloroform extraction method of Beechey *et al* (1975) was applied to the PAB-washed membranes (Scheme II.1b). The membranes were resuspended in the 5mM-Tes buffer system (containing PAB) to about 10mg protein/ml. Chloroform (½ volume) was added to the membrane suspension and the mixture shaken vigorously for 30 sec. The emulsion was broken by centrifugation, and the aqueous layer removed and centrifuged at 160,000g for 2 hours. The supernatant was then concentrated to about 4ml by ultrafiltration.

(ii) Minus-PAB wash method (Scheme II.1c)

The PAB-washed membranes were resuspended in the 5mM-Tes buffer system, omitting PAB, and centrifuged at 160,000g for 2 hours. The centrifugation was repeated, and the final membrane pellet was resuspended to give EACA-washed membranes. The supernatants were pooled and concentrated as before, to give the 'Concentrated Minus-PAB Wash'.

(c) Storage conditions

All fractions were stored frozen at -18°C, or for short times at 4°C. Mg-ATPase activity was stable under these conditions for several months at -18°C, and for at least several days at 4°C.

I. ASSAY TECHNIQUES

(a) Assay of Mg-ATPase activity

Mg-ATPase activity was assayed as described by Cox and Downie (1978). The reaction mixture which was preincubated at 30°C,

contained 0.1M-Tris-HCl buffer, pH 9.0, 20mM-ATP, and 10mM-$MgCl_2$ in a final volume of 1ml. The reaction was started by the addition of up to 20µl of enzyme preparation. After incubation at 30°C for 10 min, a 0.5ml sample was added to 9.5ml of King's Reagent (King, 1932). After 15 min, the E_{660} was measured, using a Gilford flow-through spectrophotometer. All samples were assayed at two concentrations, and at 0 min, 3 min and 6 min, to ensure that the activity was proportional to time and to the amount of protein added. The inorganic phosphate concentration was determined by comparison with standards.

(b) Assay of oxidative phosphorylation - P/O ratio

The determination of P/O ratios in unwashed membrane preparations was described by Cox *et al* (1973a). Oxygen uptake and esterification of $^{32}P_i$ were measured simultaneously at 30°C. The reaction mixture contained, in a final volume of 2.5ml, 0.1M-Tes buffer (pH 7.0), 0.25M-sucrose, 0.02M-magnesium acetate, 0.25mM-EGTA, 0.15mM-ADP, 0.75mM-AMP, 3mM-glucose, 50 EC units of hexokinase and $^{32}P_i$ to give a specific radioactivity of approximately 100c.p.m./nmol of phosphate. Membranes (about 2mg of protein) were pre-incubated with the above mixture at 30°C for 3 min, and the reaction was started by the addition of either NADH (at a final concentration of 1.2mM) or D-lactate (at a final concentration of 6mM). Oxygen uptake was measured polarographically with a Titron oxygen electrode (Titron Instruments, Melbourne, Vic., Australia) modified as described by Snoswell (1966). While oxygen uptake was linear, the reaction was stopped by transferring 1ml of the reaction mixture into 1ml of ice-cold 20% (v/v) trichloroacetic acid. Denatured

protein was removed by centrifugation, and P_i was extracted as a phospho-molybdate complex by the method of Avron (1960). A sample of the aqueous residue (2ml) was added to 8ml of water in a scintillation vial. The Čerenkov radiation was measured in a Packard liquid-scintillation counter. Incubation mixtures without added substrates were included as controls, and vials containing known concentrations of $^{32}P_i$ were used as standards.

(c) Estimation of protein

Protein concentrations were determined by the method of Lowry *et al* (1951), using a standard curve obtained with bovine serum albumin. The E_{500} was measured 30 min after the addition of Folin's phenol reagent.

J. ATEBRIN FLUORESCENCE QUENCHING TECHNIQUE

The quenching of atebrin fluorescence occurs during the energization of membranes by ATP hydrolysis or substrate oxidation (Haddock and Downie, 1974; Nieuwenhuis *et al*, 1973). The method used was modified (J. A. Downie, personal communication) from those described by Cox and Downie (1978) and Haddock and Downie (1974).

Atebrin fluorescence quenching was measured at 30°C in a fluorimeter using an activation wavelength of 450nm and measuring the light emitted at 510nm. The cuvette contained, in a volume of 2.5ml, 10mM-Hepes (N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid-KOH) (pH 7.5), 300mM-KCl, 5mM-$MgCl_2$ and 1.0-2.5mg of membrane preparation. Atebrin (final concentration 2μM) was added, and the

extent of fluorescence was recorded. NADH (final concentration 1mM) was added, and the extent of quenching of fluorescence was recorded. Cyanide (final concentration 2.5mM) was added to inhibit respiratory-dependent quenching, and when the fluorescence returned to about the initial level, ATP was added (final concentration 0.8mM). The extent of fluorescence quenching was again recorded, and carbonylcyanide-*m*-chlorophenylhydrazone (CCCP) was added at a final concentration of 4μM.

Atebrin fluorescence quenching was also measured in washed membranes reconstituted with Mg-ATPase preparations. Membranes (1.0-2.5mg) were pre-incubated for 10 min at 30°C with a preparation of Mg-ATPase (0.7-4mg) in the presence of 30mM-$MgCl_2$. The reconstituted membranes were then diluted to 2.5ml, and the fluorescence quenching measured.

K. TWO-DIMENSIONAL GEL ELECTROPHORESIS UNDER NON-DISSOCIATING CONDITIONS

The acrylamide stock solution used for the first dimension and the separating gel of the second dimension, was described by O'Farrell (1975) as 'Solution D' (see Section M(a)).

(a) First Dimension: Isoelectric Focusing

Isoelectric focusing was performed in polyacrylamide gels prepared in glass tubes (132mm long, 5mm inner diameter). The tubes were sealed at the bottom with laboratory film and placed in a rack of tightly-fitting rubber holders. A gel mixture containing 3.78%

(w/v) acrylamide, 0.22%N,N'-methylenebisacrylamide, 25%(v/v) glycerol, 1.2%(w/v) Ampholine (pH range 4-6) and 0.8% Ampholine (pH range 6-8), was prepared. 0.33% Ammonium persulphate (added as a 10%(w/v) solution) and 0.08% Temed (N,N,N',N'-tetramethylethylenediamine) were added, and the gels were poured immediately, leaving sufficient room for the sample. The gel mixture was overlaid with water, and allowed to polymerize for about one hour. The lower chamber of the electrophoresis tank (Model 150A, Biorad Laboratories, Richmond, California, U.S.A.) was filled with 10mM H_3PO_4. Unpolymerized material was removed from the tubes, the tubes were mounted in the tank, and the samples were applied to the top of the gels. The remainder of each tube, and the top chamber, were filled with 0.2%(v/v) ethanolamine. The gels were run with cooling at a constant current to give 180V initially, rising to about 500V, and this voltage was then maintained. Total running time was 9 hours. Gels were removed from the tubes by forcing water between the gel and the glass using a syringe with a thin needle. Gels were mounted on the second dimension as described below, or sliced into 5mm segments, each of which was equilibrated in 2ml water and the pH determined.

(b) Second dimension: Polyacrylamide-gradient Electrophoresis

Electrophoresis in the second dimension was performed in slab polyacrylamide-gradient gels, using a method similar to that described by Davis (1964) and apparatus similar to that described by O'Farrell (1975). The separating gel was 3mm thick, 15cm wide, and 9cm tall. Gradients (6% to 7.5% acrylamide) were poured using a standard two-chambered gradient mixer. The back chamber contained

5.68%(w/v) acrylamide, 0.32%N,N'-methylenebisacrylamide, 0.38M-Tris-HCl (pH 8.8), 2mM-$MgSO_4$,0.029% Temed, and 0.065% ammonium persulphate; the front chamber contained 7.10%(w/v) acrylamide, 0.41%N,N'-methylenebisacrylamide, 0.38M-Tris-HCl (pH 8.8), 2mM-$MgSO_4$, 0.029% Temed, 0.055% ammonium persulphate, and 18.6%(v/v) glycerol. The gradient was poured at 3ml per min through flexible tubing, the end of which was kept about 1cm above the gel front during pouring. The gel was overlaid gently with water and left to polymerize for one hour. Aluminium foil was used to insulate the gel during polymerization. The stacking gel mixture consisted of 2.5%(w/v) acrylamide, 0.63%N,N'-methylenebisacrylamide, 49mM-Tris-HCl (pH 6.8), 2mM-$MgSO_4$, 0.046% Temed, 0.031% ammonium persulphate, and 9.4%(v/v) glycerol. After the unpolymerized material was removed from the separating gel, the stacking gel was poured and left to polymerize for about an hour. The unpolymerized material was again removed, and the focusing gel from the first dimension was mounted on top of the slab gel. Agarose was dissolved to 1.5%(w/v) in boiling electrode buffer, then cooled to about 40^{o}C and poured around the focusing gel to form a seal. After the agarose gel had set, the electrode buffer, containing 5mM-Tris, 38.4mM-glycine, and 2mM-$MgSO_4$, was added to both chambers, and the current was set at 20mA for 13-16 hours. Mg-ATPase activity was detected as a white precipitate in the gel following incubation at room temperature in a buffer containing 35mM-Tris, 270mM-glycine, 14mM-$MgSO_4$, 0.2% $Pb(NO_3)_2$, and 8mM-ATP. The gels were photographed using a diffuse light source, with filters of high neutral density (Polaroid NH38, Polaroid Corporation, Cambridge, Massachusetts, U.S.A.) between the light source and the gel, and at the lens. The area(s) of gel containing Mg-ATPase activity were excised carefully with a razor blade, usually

after immersion in the lead stain for a minimal time.

L. SOLUBILIZATION OF POLYPEPTIDES

Proteins were solubilized and dissociated into polypeptide chains using SDS (sodium dodecyl sulphate). The solubilizing buffer contained 40mM-Tris-HCl (pH 6.8), 9.6%(v/v) glycerol, 1.6%(w/v) SDS and 4%(v/v) 2-mercaptoethanol. 1 volume of this buffer was heated to 100°C in a water bath, and 2 volumes of the sample added and mixed rapidly, to give a final protein concentration of 2-5mg/ml. After 4 min, the solubilized protein was removed from the heat, and stored frozen at -18°C.

Pieces of gel containing Mg-ATPase (see Section K) were incubated in the boiling SDS buffer for 5 min.

M. TWO-DIMENSIONAL GEL ELECTROPHORESIS UNDER DISSOCIATING CONDITIONS

The method of O'Farrell (1975) was used, with modifications as described below. For clarity, the full method is described using the notation of O'Farrell (1975).

(a) Buffers and Solutions

The following buffers and solutions were described by O'Farrell (1975):

(D) 30% acrylamide stock for isoelectric focusing gels:

28.38%(w/v) acrylamide and 1.62%(w/v)N,N'-methylenebisacrylamide.

(E) Stock Nonidet P-40 solution: 10%(w/v) NP-40 in H_2O.

(F) Ampholines were used as supplied: 40%(w/v) solutions, except for pH range 9-11 (20%(w/v) solution).

(G) Ammonium persulphate: a fresh 10%(w/v) solution was prepared every week.

(H) Gel Overlay Solution: 8M-urea, stored as frozen aliquots.

(I) Anode Electrode Solution: 10mM H_3PO_4.

(J) Cathode Electrode Solution: 20mM NaOH (or 0.2M-NaOH).

(K) Sample Overlay Solution: 9M-urea, 1% Ampholines (pH range 3.5-10).

(L) Separating Gel Buffer: 1.5M-Tris-HCl (pH 8.8) and 0.4% SDS.

(M) Stacking Gel Buffer: 0.5M-Tris-HCl (pH 6.8) and 0.4% SDS.

(N) 30% acrylamide stock for SDS gels: 29.2%(w/v) acrylamide and 0.8% N,N'-methylenebisacrylamide.

(Q) Electrode Buffer: 25mM-Tris base, 192mM-glycine, and 0.1% (w/v) SDS.

Other buffers used were:

(A') Solubilizing buffer: (See Section L).

(L') Buffer (L) made up in 50%(v/v) glycerol.

(O') SDS equilibration buffer: Buffer M was diluted 4-fold, and SDS concentration increased to 2%(w/v).

(P') Agarose gel: 1%(w/v) agarose melted in buffer Q.

(T') Fixing solution: 5%(w/v) trichloroacetic acid, 5%(w/v) sulphosalicylic acid, and 10%(v/v) methanol. The sulphosalicylic acid was dissolved in the methanol before water and trichloroacetic acid were added.

(U') Staining solution: 0.12% Coomassie blue R250 in solution (V').

(V') Destaining solution: 25%(v/v) ethanol and 8%(v/v) acetic acid.

(W') Agarose gel: 1.5%(w/v) agarose melted in 2:1 (solution K:H_2O).

Buffers A', L' and O' were stored at 4°C, Solution T', U' and V' were stored at room temperature, and storage conditions for other solutions are given by O'Farrell (1975). Acrylamide stock solutions were kept for a maximum of 3 weeks.

(b) First dimension: Isoelectric Focusing

Cylindrical gels were prepared in glass tubes (132mm long, 5mm inner diameter). The gel mixture (10ml per 4 tubes) was made in a 125ml side-arm flask. Solid urea (5.5g) was dissolved in 1.97ml of H_2O, 2.0ml of NP-40 stock (E), 1.33ml of acrylamide stock (D), and 0.5ml (total) Ampholines. The Ampholine composition was either (i) 1:1:3 (pH ranges 3.5 to 10: 5 to 7: 4 to 6) or (ii) 1:4 (pH ranges 3.5 to 10: 4 to 6), or (iii) 1:1:1:2 (pH ranges 3.5 to 5: 5 to 7: 7 to 9: 9 to 11), The solution was de-gassed under vacuum until all the bubbles had disappeared. Ammonium persulphate (10µl of G) and 7µl Temed were added, and the gel mixture was loaded carefully into the tubes. These amount of catalysts were doubled for pH range (iii). Solution H was layered carefully over the gel mixture, which was left for 1 hour to polymerize. After the overlay solution was removed, the gels were mounted in the electrophoresis tank (Model 150A, Biorad Laboratories, Richmond, California, U.S.A.) to which 1 litre of 10mM-H_3PO_4 had been added. The sample (30µg to 200µg of protein) was prepared as described in Section L., and applied together with solid urea (about 10 mg per 20µl of sample), to the surface of the gel. Liquid samples were overlayed carefully with 25µl of the sample overlay solution (K) and

then with the cathode solution. Samples of polyacrylamide gel were sealed to the isoelectric focusing gel using hot agarose gel (W'). The upper (cathode) chamber was filled with 20mM-NaOH for pH ranges (i) and (ii), or 0.2M-NaOH for pH range (iii). Gels were not pre-run; samples were electrophoresed at constant current for 3 hours to give 180V initially, rising to about 500V, and then at 600V for a further 3 hours.

Gels were removed from the tubes by forcing water between the gel and the glass using a syringe with a thin needle. Gels used for pH gradient measurement were sliced into 5mm sections, each of which was equilibrated in 2ml of water for pH determination. Gels to be electrophoresed in the second dimension were shaken in SDS equilibration buffer (O') for about 15 min, before they were frozen or applied to the second dimension slab gel.

(c) Second dimension: Gradient Electrophoresis in SDS

The apparatus used was similar to that described by O'Farrell (1975). The separating gel was 1.5mm thick, 15cm wide and 9cm tall. A gradient of 7.5% to 22.5% acrylamide was poured using a standard two-chambered gradient mixer. The back chamber contained, in a volume of 11ml, 2.75ml of acrylamide stock (N), 2.75ml of separating gel buffer (L), 5.5ml of H_2O, 35µl of ammonium persulphate solution (G), and 7µl of Temed. The front chamber contained, in a volume of 11ml, 8.25ml of acrylamide stock (N), 2.75ml of separating gel buffer in glycerol (L'), 15µl of ammonium persulphate solution (G), and 3µl of Temed. The gradient was poured at 3ml per min through flexible tubing, the end of which was kept about 1cm above the gel front during pouring. The gel was overlaid

gently with water, and left to polymerize for one hour. Aluminium foil was used to insulate the gel during polymerization. The stacking gel mixture was prepared by mixing together: 2.5ml of stacking gel buffer (M), 1.5ml of acrylamide stock (N), 6ml of H_2O, 30µl of ammonium persulphate solution (G) and 10µl of Temed. After the unpolymerized material was removed from the separating gel, the stacking gel was poured, overlaid with H_2O, and left to polymerize for about an hour. A mould was used to form wells for single-dimensional runs. De-gassing of the acrylamide solutions used in the second dimension was found to be unnecessary.

The focusing gel was sealed to the slab with hot agarose gel (P'), after the unpolymerized material had been removed from the slab surface, After the agarose gel had set, the electrode buffer (Q) was added to both chambers. Gels were run at 14mA for 12 hours (single-dimensional gels) or 13.5 hours (two-dimensional gels), without a dye marker.

(d) Fixing and staining of proteins

Proteins were fixed in the gel slab by incubation at 60°C for 30 min in fixing solution (T'). The gels were then stained for 60 min at room temperature in staining solution (U') and destained in several changes of destaining solution (V').

The same procedure could be used for staining the gel slabs described in section (K), or for isoelectric focusing gels containing small amounts of protein.

The stained gels were photographed through a yellow filter, which increased the contrast between faint spots and the surrounding gel.

Chapter III

TWO-DIMENSIONAL ANALYTICAL GEL ELECTROPHORESIS OF MEMBRANE POLYPEPTIDES

A. INTRODUCTION

As discussed in Chapter I, mutations in the *unc* genes affect the membrane-bound Mg^{2+}-stimulated adenosinetriphosphatase (Mg-ATPase) complex. Since the lesions caused by such mutations are likely to be in subunits of the Mg-ATPase, it was desirable to have a method of analysis of the membrane polypeptide components, capable of detecting alterations in the polypeptides constituting the membrane-bound Mg-ATPase. A method which appeared to be suitable was the two-dimensional electrophoresis procedure of O'Farrell (1975), which gave high resolution of the polypeptides of a complex mixture of proteins, and moreover was sufficiently sensitive to detect a single charge alteration in a polypeptide, such as that caused by some missense mutations. In this procedure, proteins are separated according to their isoelectric points by isoelectric focusing in the first dimension, and according to their molecular weights by SDS (sodium dodecyl sulphate) electrophoresis in the second dimension. These two parameters are unrelated, and polypeptides are therefore distributed widely over the resulting two-dimensional gel. Before electrophoresis, the proteins are dissociated into discrete polypeptide chains using a buffer containing urea, mercaptoethanol, and a non-ionic detergent (Nonidet P-40). Using this technique, O'Farrell (1975) was able to distinguish 1100 polypeptides on a two-dimensional gel of a preparation of proteins from whole *E.coli*.

This chapter describes the development of a procedure for the analysis of the polypeptide composition of membrane preparations, based on the method of O'Farrell (1975).

B. REFINEMENT OF THE PROCEDURE FOR TWO-DIMENSIONAL ANALYTICAL ELECTROPHORESIS

(a) Isoelectric focusing in flat-bed gels

A flat-bed electrophoresis tank suitable for isoelectric focusing (LKB Multiphor, LKB Produkter, Bromma, Sweden) was available, and attempts were made to use this apparatus for the first dimension isoelectric focusing. In the O'Farrell technique, cylindrical isoelectric focusing gels were used. Several problems were encountered during experiments using the flat-bed system. Firstly, the urea concentration within the gel had to be lowered from 9.5M to 8M, to avoid the formation of urea crystals during polymerization of the gel. Secondly, no acceptable method of sample application could be found. Direct application of the sample, or an agarose gel containing the sample, to the gel surface, required very high concentrations of protein in the sample. Wells set in the gel resulted in unacceptable distortion of the isoelectric focusing, even when the thickness of the gel slab was increased from 1mm to 3mm. Indirect application of the sample, using filter paper (LKB); or Whatman 3MM or No. 3, Whatman Ltd., Maidstone, Kent, U.K.) or dried acrylamide gel, was unsatisfactory due to the high retention of protein by these support media. No improvement was noted when the sample was applied to filter paper which had been

soaked in a 2%(v/v) Ampholine solution and then dried. Ampholines are synthetic polyamino-polycarboxylic acids used to form a stable pH gradient during isoelectric focusing (see Righetti and Drysdale, 1976, especially pp. 357-372) and should react with groups which bind proteins. It was also found that virtually no proteins travelled from filter paper supports toward the cathode, necessitating the application of samples near the cathode wick. When a suitable cylindrical-gel apparatus became available, the flat-bed electrophoresis system was abandoned.

(b) Two-dimensional electrophoresis by the method of O'Farrell

Isoelectric focusing in cylindrical gels was performed by the method of O'Farrell (1975) using samples of an aqueous extract from membranes. Five identical first-dimensional gels were run, and one was subjected to second dimension electrophoresis in a slab gel containing 7%(w/v) acrylamide, made by the method of O'Farrell (1975) (Fig. III.1). The remaining isoelectric focusing gels were stained for protein (Fig. III.1). However, when the method was applied to the separation of membrane proteins, serious drawbacks were encountered. The problems experienced included poor focusing of polypeptides, particularly within alkaline pH gradients, the retention and detection of low molecular weight proteins, and the formation of stable alkaline pH gradients. These problems, together with the measures adopted to overcome or partially overcome them, are now discussed.

(c) Measures taken to overcome the problems encountered during electrophoresis of membrane proteins

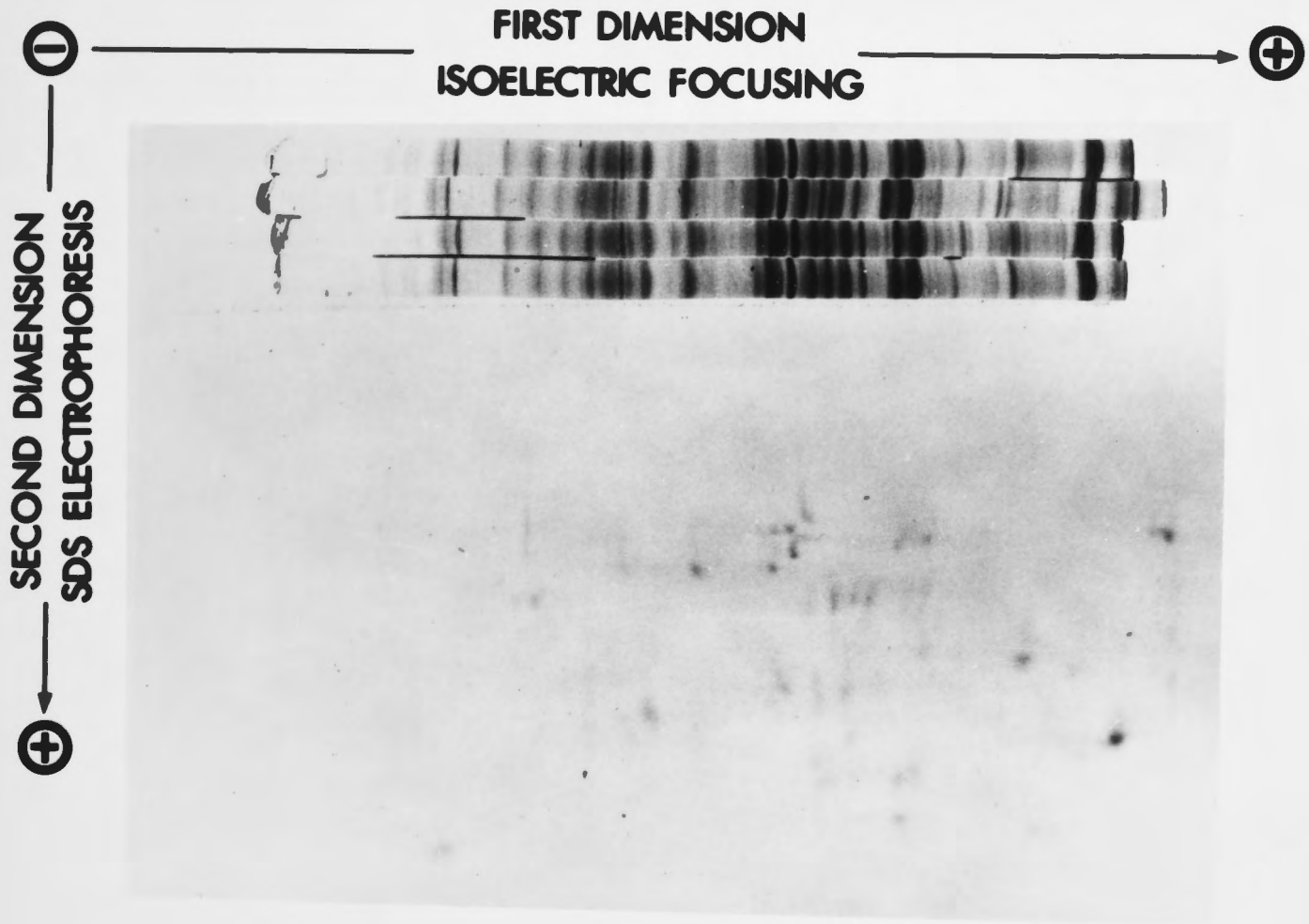


Figure III.1 Two-dimensional analytical gel electrophoresis by the method of O'Farrell (1975). The sample applied was solubilized in Lysis buffer (buffer A of O'Farrell, 1975) which includes urea, Nonidet P-40 and β-mercaptoethanol. Five identical isoelectric focusing gels were run. The Ampholine composition (by volume) of the isoelectric focusing gels was 1:4 (pH ranges 3.5 to 10: 5 to 7). First dimension gels were pre-run at 200V for 15 min, 300V for 30 min, and 400V for 30 min, and then run at 400V for 17 hours and 800V for 1 hour. One gel was equilibrated in buffer 0 (see section B(c)(iii)), sealed using an agarose gel made in buffer 0 to a slab gel containing 7% acrylamide, and subjected to second dimension SDS electrophoresis. The remaining four isoelectric focusing gels were stained and placed on the stacking gel of the slab for photography. The SDS gel was pre-run for 3 hours at 40mA, and run for 6 hours at 20mA. The bottom section (about 1cm) of this gel was not included in the photograph. The sample consisted of concentrated fractions from a Biogel column,which contained pyrophosphatase activity.

(i) Use of acrylamide gradients in the second dimension.

When slab gels containing 7% acrylamide were used (e.g. Fig. III.1), the ion front formed during electrophoresis in the second dimension was very uneven. The spots formed by polypeptides which ran to within two centimetres of the front were severely distorted. The Ampholines could not penetrate the areas of distortion, and formed a contorted front around such areas, which may be occupied by mixed micelles of Nonidet P-40 and SDS (see O'Farrell, 1975).

The problem was somewhat reduced by the introduction of gel slabs containing a gradient of 7.5% to 18% acrylamide (Fig. III.2). By increasing the acrylamide concentration at the bottom of the gel still further, and increasing the duration of electrophoresis, the problem of an uneven ion front was eliminated. O'Farrell (1975) had found that slab gels with or without an acrylamide gradient could be used. However, in my hands, slab gels containing 7% or 10%(w/v) acrylamide, even when pre-run (Fig. III.1), were unsuitable, especially for polypeptides below a molecular weight of 25,000. A gradient of 7.5% to 22.5% acrylamide was used for subsequent experiments.

(ii) Revised staining procedure.

The gels shown in Fig. III.1 were stained by the method of O'Farrell. Areas containing Ampholines stained strongly, and several weeks of destaining were needed before complete destaining occurred. Occasionally, the Ampholines could not be destained (Fig. III.2).

Numerous staining procedures were tried, including those of O'Farrell (1975) and Ames and Nikaido (1976). The only procedure found to be entirely satisfactory, was based on a method

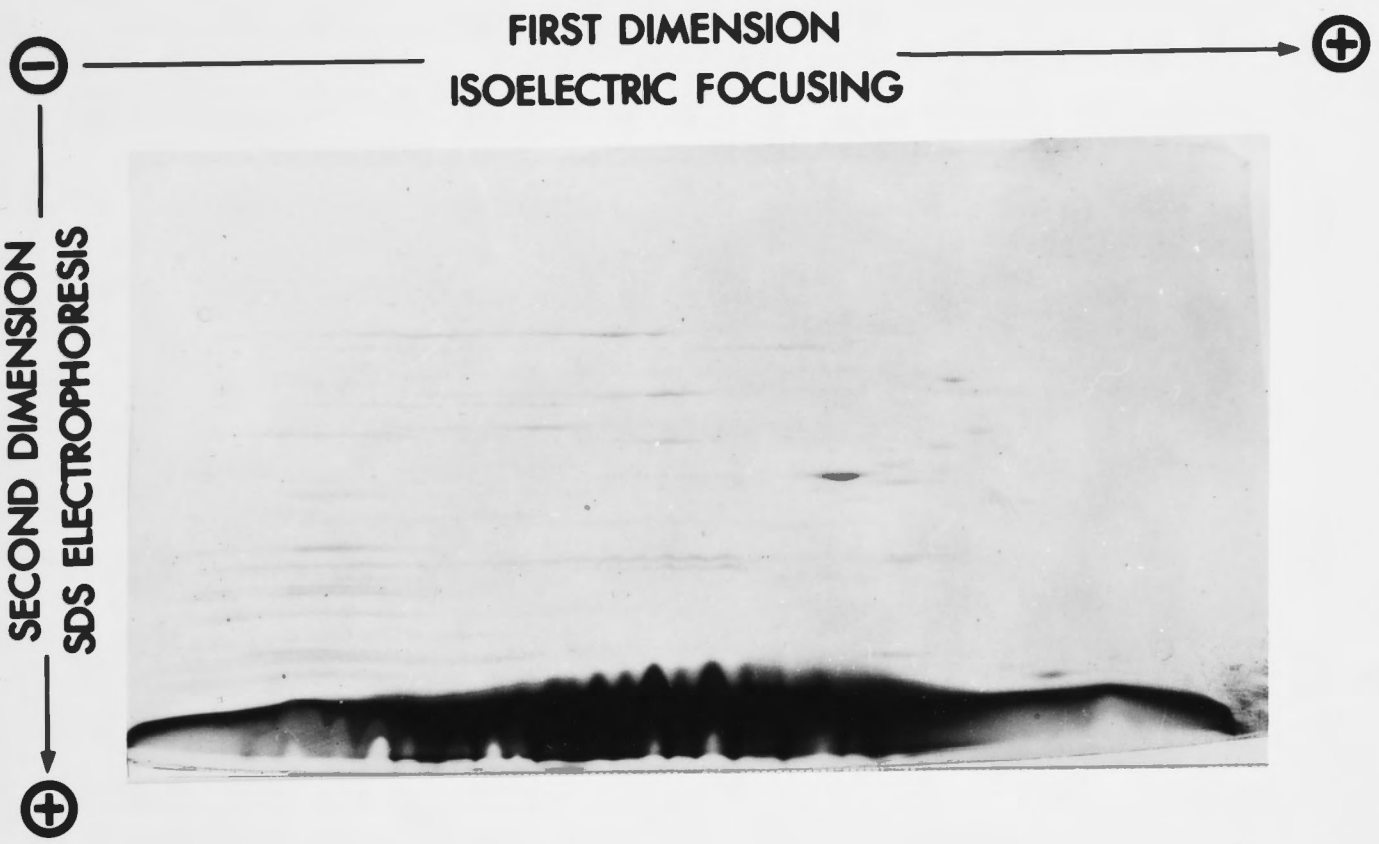


Figure III.2 Two-dimensional analytical gel electrophoresis of washed membranes from strain AN716 (*uncB402*). Washed membranes were prepared as described by Cox *et al* (1973a), and were solubilized in lysis buffer (buffer A of O'Farrell, 1975) which includes urea, Nonidet P-40 and βmercaptoethanol. The isoelectric focusing gel contained Ampholines consisting of 1:1:1:1 (by volume) of pH ranges 3.5 to 5: 5 to 7: 7 to 9: 9 to 11. The electrode solutions were 0.2M-NaOH (cathode) and 0.1M-H_3PO_4 (anode), and the gel was run at 200V for 15 min, 300V for 10 min, 400V for 15 min, 500V for 15 min, and 600V for 3 hours. The isoelectric focusing gel was equilibrated in SDS equilibration buffer (see Chapter II.M) but was sealed to the slab gel using the agarose gel of O'Farrell (1975) (see section B(c)(iii)). The SDS gel contained a gradient of 7.5% to 18% acrylamide, and was run at 14mA for 12 hours. The gel was not fixed before staining with Coomassie blue. The pH gradient formed in the first dimension extended from pH 10.2 to 3.9 (left to right) and was almost linear.

recommended in a pamphlet circulated by LKB. The staining and destaining steps are as described in Chapter II.M(d). The LKB fixing solution contained 12% sulphosalicylic acid. The fixing solution was modified to include 5% trichloroacetic acid and 5% sulphosalicylic acid, on the basis of a report by Bachorik *et al* (1974) in which they found that fixing in a solution containing both acids prevented the leaching of low molecular weight proteins from gels. In agreement with their findings, the use of the modified fixing solution (Chapter II.M(d)) resulted in increased intensities of staining of low molecular weight polypeptides. Ampholines were destained satisfactorily using the revised procedure, allowing low molecular weight polypeptides to be distinguished.

(iii) Prevention of protein loss from the isoelectric focusing gel before second dimension electrophoresis.

A comparison of the isoelectric focusing gels of Fig. III.1 with the corresponding two-dimensional gel, reveals that a considerable amount of protein has been lost.

A combination of two alterations to the procedure, eliminated the problem of loss of protein between first and second dimension electrophoresis. Firstly, an altered procedure was introduced for the equilibration of the isoelectric focusing gel in preparation for second dimension electrophoresis. Both glycerol and mercaptoethanol were omitted from the equilibrating buffer (Buffer 0 of O'Farrell, 1975, which is buffered at pH 6.8 and also contains 2.3%(w/v) SDS). Complete equilibration took only 15 min, and the longer equilibration times recommended by O'Farrell (1975) tended to result in the leaching of low molecular weight proteins from the

gel. Secondly, in the original method the isoelectric focusing gel was sealed to the slab using an agarose gel made up in fresh Buffer O (pH 6.8). The revised procedure used an agarose gel made up in the electrode buffer (Q;Chapter II.M(a)) which contains only 0.2% SDS and is buffered at pH 8.3. A combination of the two amendments was found to improve significantly the transfer of protein to the second dimension.

(iv) The use of SDS to solubilize membrane proteins

The sample used for the gels of Fig. III.1 was an aqueous extract of membranes, treated with the lysis buffer of O'Farrell (1975). Much poorer results were obtained with membrane preparations, and many proteins streaked badly in the first dimension. The problem of streaking was particularly severe when alkaline pH ranges were used (Fig. III.2).

The streaking of polypeptides in the neutral and alkaline regions of the isoelectric focusing gels appeared to be a problem of solubilization rather than electrofocusing, since the pH gradients formed were reproducible and almost linear. At this stage, Ames and Nikaido (1976) reported that the solubilization procedure used by O'Farrell (1975) failed to solubilize many membrane proteins, but that most of these proteins were solubilized during treatment with SDS. Miller & Elgin (1974) and O'Farrell (1975) had found that the addition of SDS to the sample before isoelectric focusing did not destroy the separation, and Ames & Nikaido (1976) confirmed and extended these findings. A procedure for solubilization in SDS was therefore tried. Proteins were treated with SDS + mercaptoethanol at 100^{o}C for 3 min, as described in

Chapter II.L. Solid urea (to give a concentration of about 8M) was added to the sample in the electrophoresis tube.

Although this solubilization procedure reduced the extent of streaking of proteins it did not completely eliminate the problem. O'Farrell (1975) found that treatment of his samples with DNAase and RNAase reduced streaking in the neutral region. In the present work, treatment of the membrane samples with nucleases by the method described by O'Farrell (1975) and variations of it, had little or no effect on the polypeptide patterns obtained.

(v) Focusing at alkaline pH

Preliminary experiments with two-dimensional electrophoresis of partially-purified Mg-ATPase, indicated that the γ-subunit had a very alkaline isoelectric point. The gels of Fig. III.1 covered a pH range of about 5 to 7 only (measured in de-gassed water; see O'Farrell, 1975), whereas the first-dimension gel of Fig. III.2 contained an almost-linear pH gradient from pH 10 to 4.

Considerable effort was devoted towards obtaining acceptable isoelectric focusing over a wide pH range, ideally pH 4.5 to 11, to include essentially all of the membrane proteins. No completely satisfactory method was obtained. The original method (O'Farrell, 1975) required pre-focusing of the Ampholines before the application of the sample, followed by focusing for 13 hours. When this method was applied directly to gels containing Ampholines covering pH ranges between 7 and 11, the ends of the pH gradient were lost. For instance, a gel containing Ampholines of pH range 3.5 to 10 formed an almost linear pH gradient from pH 8.8 to 4.5. The loss of the acidic end of the gradient presented no problem, since no

proteins are found below pI 4.8 (see also Ames & Nikaido, 1976). The absence of the alkaline end however, resulted in the loss of many potentially interesting polypeptides, amongst which was the γ-subunit of the Mg-ATPase. The loss of Ampholines of high pI, otherwise known as 'cathodic drift', is a general phenomenon (see Righetti & Drysdale, 1976, pp. 525-6).

Three distinct amendments to the original method were required to overcome the loss of the alkaline pH ranges. Firstly, the strength of both electrode buffers was increased ten-fold. The increase in the strength of the anode buffer was later found to be unnecessary. Many cathode buffers were tested, including 10%(v/v) ethanolamine, 50mM-NaOH + 5% ethanolamine, or 0.2M-NaOH with various amounts of Ampholine (pH ranges 9 to 11 and 7 to 9). All such buffers allowed substantial losses of the alkaline pH range, and only the use of the last-mentioned buffer gave an occasional marginal improvement in focusing, which did not justify the expense involved. Secondly, the pre-running of isoelectric focusing gels was found to be detrimental, and was omitted. Thirdly, the total time taken for focusing was decreased. Gels were run for only 6 hours, instead of the 13 hours required by the original method.

A further problem arose when Ampholines of high pI were included in the gel mixture for the first dimension. Gels failed to polymerize completely and evenly. This problem was overcome by increasing the concentrations of both catalysts (ammonium persulphate and Temed) (see Chapter II.M(b)).

C. TWO-DIMENSIONAL ELECTROPHORESIS USING ACETIC ACID-UREA GELS IN THE FIRST DIMENSION

While the isoelectric focusing gels used in the first

dimension of the O'Farrell technique were proving troublesome (see Section C), attempts were made to replace them with the acetic acid-urea gel system of Takayama *et al* (1964). Cylindrical gels containing 7.5%(w/v) acrylamide, 0.2%N,N'-methylenebisacrylamide, 5M-urea, and 35%(v/v) acetic acid were prepared as described by Takayama *et al* (1964), except that all of the urea was added as a solid to the gel mixture, and dissolved before the catalysts were added. Samples were usually acetone powders dissolved in phenol/acetic acid/water (2:1:1, w/v/v), and the gels were prepared and run as described by Takayama *et al* (1964) except that the current was set at 1mA per tube for 16 hours.

It was found that the acetic acid-urea gels must be very well equilibrated in SDS equilibration buffer (Chapter II.M(a), buffer 0') before proteins move into the second dimension. The procedure adopted was to wash the gel thoroughly in water, and then to shake the gel in several changes of SDS equilibration buffer followed by incubation overnight before electrophoresis in the second dimension.

Examples of the patterns obtained using this two-dimensional procedure are shown in Fig. III.3. Samples of an aqueous extract from membranes of strain AN295 (see Table II.1) were treated with phenol-acetic acid (Takayama *et al*, 1964) and subjected to electrophoresis. Twice as much protein was applied to the gel shown in Fig. III.3b as compared with that in Fig. III. 3a. Most of the proteins in the gels of Fig.III.3a lie roughly along a curved line. A comparison of the degrees of mobility in each dimension reveals that many adjacent pairs of proteins have reversed orders of mobility, presumably due to discrepancies between their total charges and their SDS-binding capacities. A

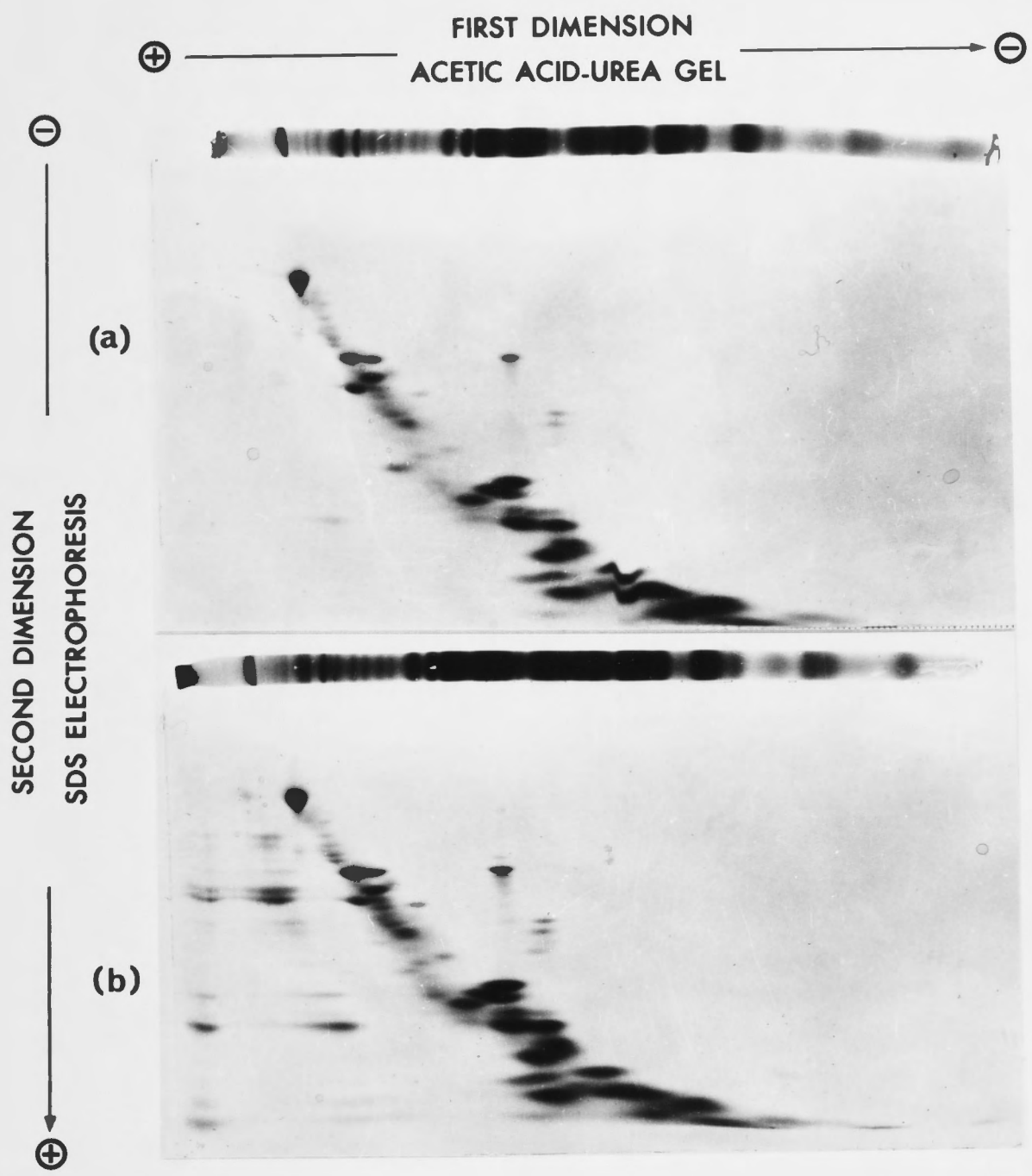


Figure III.3. Two-dimensional gel electrophoresis using acetic acid-urea gels in the first dimension. Duplicate acetic acid-urea gels were run at 1mA per tube for 16 hours. One pair of gels (a) contained about 0.5mg of protein, and the other pair (b) contained about 1mg of protein. The concentrated EACA wash (see Chapter IV) from strain AN295 (Cox *et al*, 1973a) was dissolved in an equal volume of phenol/acetic acid/water (4:2:1, w/v/v). One of each pair of first-dimension gels was stained with 0.5% Amido black for 30 min and then destained in 10% acetic acid (Takayama *et al*, 1964), and the other gel was washed with several changes of SDS equilibration buffer (0.125M-Tris-HCl, pH 6.8 containing 2%(w/v) SDS), and left in the same buffer for 21 hours. The SDS separating gels contained 7% acrylamide, and were pre-run for 2.5 hours at 40mA, and then run for (a) 5.3 hours, and (b) 5 hours, at 20mA.

few polypeptides are found well above the diagonal, suggesting that some proteins which dissociated in the phenol-acetic acid-urea system and remained dissociated during first dimensional electrophoresis, formed some sort of aggregate in the SDS system. Conversely, the proteins which are found below the diagonal appear to be aggregates in the first dimension which dissociate in SDS. The latter aggregates appear to be formed principally when high sample loads are applied (Fig.III.3b).

D. THE METHOD USED FOR TWO-DIMENSIONAL ANALYTICAL ELECTROPHORESIS OF MEMBRANE POLYPEPTIDES

When acetic acid-urea gels were used for first-dimension electrophoresis, the majority of polypeptides fell roughly along a diagonal line in the second dimensional SDS gel. Senior and MacLennan (1970) found that the mobility of proteins in the gel system of Takayama *et al* (1964) was proportional to the logarithm of their molecular weight. Separation in SDS gels is also proportional to the logarithm of molecular weight (see e.g. Fig. V.5), hence the 'diagonal' pattern obtained after two-dimensional electrophoresis.

Since this two-dimensional electrophoresis procedure provided considerably less resolution than the O'Farrell method, it was considered to be of little use in the present studies, and subsequent work utilized the O'Farrell technique, modified as described in this chapter. The final method is described in full in Chapter II.L and M. Samples were dissociated in SDS/mercaptoethanol at 100^{o}C, and urea was added to the sample just before isoelectric

focusing. The isoelectric focusing gels (total acrylamide (T)=4%, of which the percentage cross-linking (C)=5.7%) contained 9.5M-urea, 2%(w/v) Nonidet P-40 and 2%(w/v) Ampholines made up of one of three compositions: for acidic pH ranges in the first dimension, the Ampholine composition was either (i) 1:1:3 (pH ranges 3.5 to 10: 5 to 7: 4 to 6) of (ii) 1:4 (pH ranges 3.5 to 10: 4 to 6); or for a wide pH range (iii), Ampholines of the composition 1:1:1:2 (pH ranges 3.5 to 5: 5 to 7: 7 to 9: 9 to 11) were used. The strength of the cathode buffer and the amounts of catalysts were increased where necessary (see Chapter II.M(a)). Gels were not pre-run, and the 6-hour schedule was used for all three pH ranges, thus achieving a significant reduction in the duration of experiments. Examples of the pH gradients formed using all three pH ranges are shown in Fig. III.4.

The first dimension gels were shaken for 15 min in 125mM-Tris-HCl (pH 6.8) buffer containing 2%(w/v) SDS, and then sealed to the second dimension slab using a 1% agarose gel made up in Tris-glycine buffer (pH 8.8) containing 0.2% SDS. Second dimension SDS electrophoresis was performed in a slab gel containing a gradient of acrylamide (T=7.5 to 22.5%, C=2.67%). Gels were run for 13.5 hours at 14mA.

Before staining for protein using Coomassie blue, the slab gels were fixed at 60^{o}C for 30 min in a solution containing 5%(w/v) trichloroacetic acid, 5%(w/v) sulphosalicylic acid, and 10%(v/v) methanol.

Examples of the polypeptide profiles produced by two-dimensional electrophoresis over pH ranges (i) and (iii) are shown in Fig. III.5.

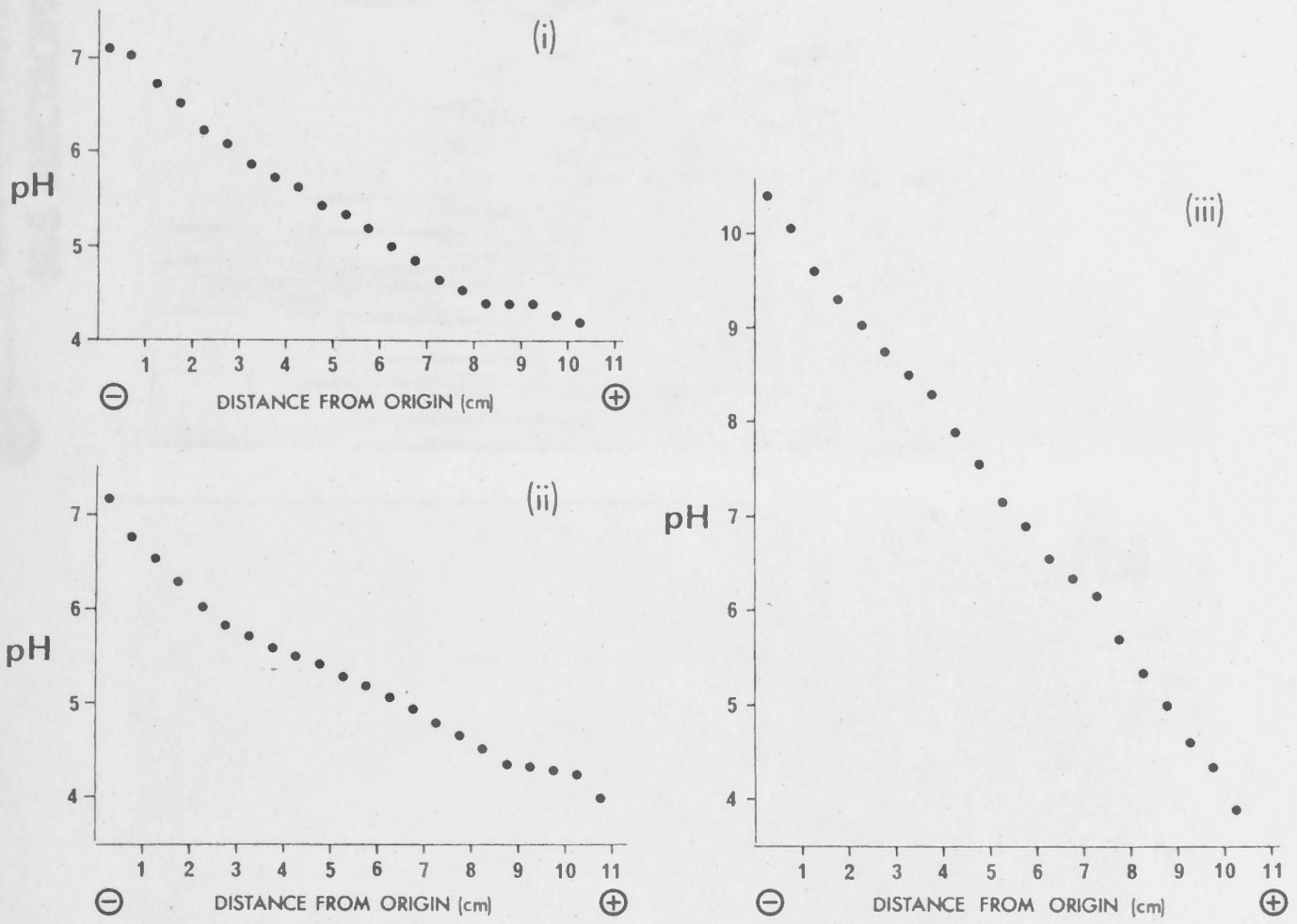


Figure III.4. The pH gradients formed during analytical isoelectric focusing in gels containing various Ampholine compositions. The ratios of Ampholines by volume were: (i) 1:1:3 (pH ranges 3.5 to 10: 5 to 7: 4 to 6); (ii) 1:4 (pH ranges 3.5 to 10: 4 to 6); and (iii) 1:1:1:2 (pH ranges 3.5 to 5: 5 to 7: 7 to 9: 9 to 11). Isoelectric focusing was performed for 3 hours at constant current to give initially 180V, followed by 3 hours at 600V. The anode solution was 0.01M-H_3PO_4, and the cathode solution was either 0.02M-NaOH (i) and (ii), or 0.2M-NaOH (iii). Gradients were measured by slicing the gels into 5mm segments, shaking each segment in 2.5ml of water, and reading the pH of the resulting solution.

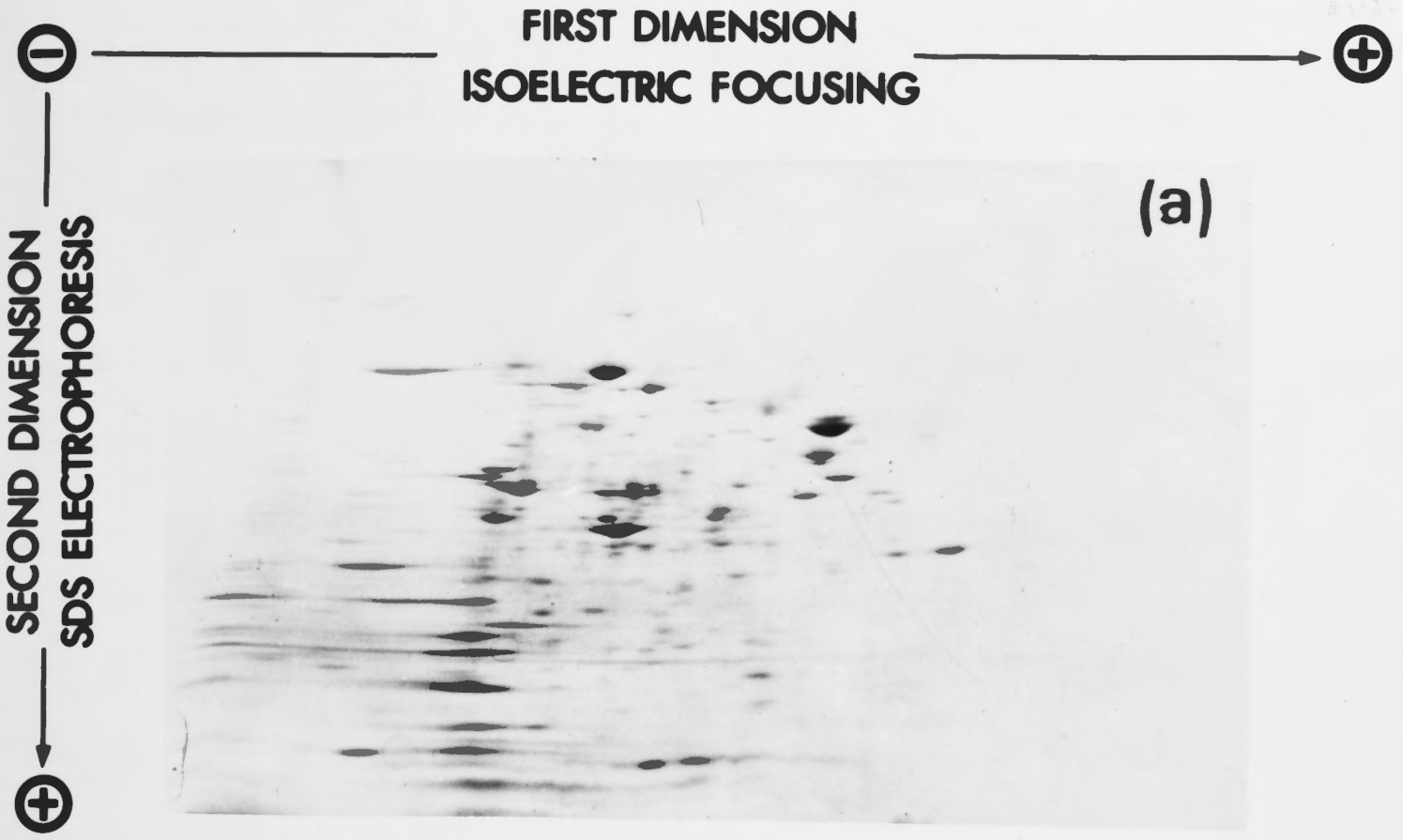


Figure III.5 Two-dimensional analytical gel electrophoresis of membrane preparations over the acidic pH range (a) and the wide range (b), using the procedure finally adopted (see section D). (a) The Ampholine composition used was 1:1:3 (pH ranges 3.5 to 10: 5 to 7: 4 to 6); the sample contained a mixture of the unwashed membranes (see Chapter IV) from strains AN248 (101µg) and AN1007 (100µg). (b) The Ampholine composition used was 1:1:1:2 (pH ranges 3.5 to 5: 5 to 7: 7 to 9: 9 to 11), and the cathode buffer was 0.2M-NaOH. In each case the sample was solubilized at 100°C for 3 min in SDS + mercaptoethanol. Fixing and staining of the SDS slab gel was carried out as described in Chapter II.M(d).

E. DISCUSSION

The two-dimensional electrophoresis procedure of O'Farrell (1975) has proved to be an excellent method for the analysis of the composition of complex mixtures of polypeptides. However, during attempts to analyse the polypeptide compositions of membrane fractions, several problems were encountered using the original method. The problems of loss of protein from gels, distortion of low molecular weight polypeptide spots, and inadequate destaining of Ampholines, were essentially overcome. The problems of streaking of proteins in the first dimension, and of 'cathodic drift' were considerably reduced. The amended procedure for two-dimensional electrophoresis was used in the work to be described in the remainder of this thesis.

During the course of this work, a method of two-dimensional electrophoresis was developed, using acetic acid-urea gels in the first dimension. The method may be useful in studies of some hydrophobic proteins, but was not used in the work to be described in this thesis. A potentially useful development might be the use of isoelectric focusing (dissociating or non-dissociating conditions) followed by second dimension electrophoresis in acrylamide gradient gels containing acetic acid-urea. Such gels would provide an alternative if the O'Farrell system was inappropriate.

Chapter IV

SOLUBILIZATION AND PURIFICATION OF THE Mg-ATPase

A. INTRODUCTION

As discussed in Chapter III, to detect possible alterations in the subunits of the Mg-ATPase in *unc* mutant strains, it was necessary to have some means of identifying the subunit polypeptides in membrane fractions. As a first step in the identification of each subunit on two-dimensional gels, it was necessary to prepare highly-purified Mg-ATPase.

Published methods used for the purification of Mg-ATPase activity from the cytoplasmic membrane of *E.coli* were discussed in Chapter I. Most of these methods rely on the initial solubilization of Mg-ATPase activity by treatment of the membranes with low-ionic strength buffers, a method of solubilization which was first reported by Pinchot (1953) using the bacterium *Alcaligenes faecalis*, and later used by Abrams (1965) to characterize the enzyme from the bacterium *Streptococcus faecalis*. Other agents which have been used to solubilize Mg-ATPase activity from *E.coli* are SDS (Evans, 1970) and Triton X-100 (Hanson and Kennedy, 1973).

The first section of this chapter summarizes the development of membrane fractionation techniques which permit the solubilization of Mg-ATPase to give preparations of relatively high specific Mg-ATPase activity. The remainder of the chapter describes the development of a two-dimensional gel electrophoresis system for the purification of solubilized, intact Mg-ATPase.

B. FRACTIONATION OF MEMBRANES AND SOLUBILIZATION OF Mg-ATPase

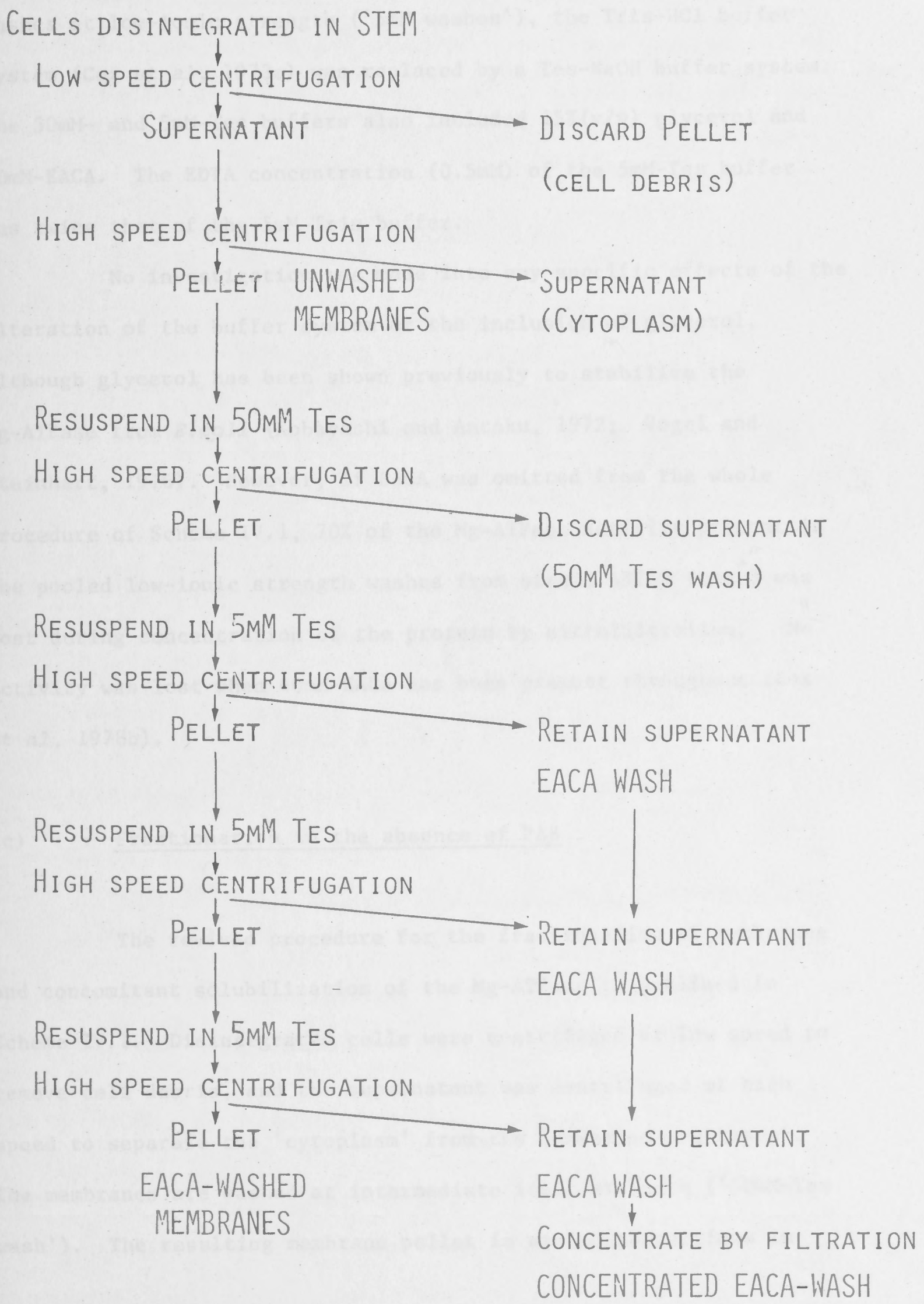
(a) Introduction

The procedures to be described below for the fractionation of membranes and the solubilization of the Mg-ATPase are based on the observation that washing of membrane preparations at low-ionic strength in the absence of Mg^{2+} ions results in the release of Mg-ATPase activity (Abrams, 1965; Cox *et al* 1973a). Recently, the protease inhibitor *p*-aminobenzamidine (Mares-Guia and Shaw, 1965) has been shown to prevent such solubilization of Mg-ATPase activity (Cox *et al*, 1978b). When 6mM *p*-aminobenzamidine (PAB) was present throughout the fractionation of normal membranes, no Mg-ATPase activity was found in the soluble fraction. This observation proved very useful, both in the purification of the Mg-ATPase to be described in this chapter, and in the investigations into the gene-polypeptide relationships in several mutants, to be described in the succeeding chapters. The effect of a second protease inhibitor, ε-amino-n-caproic acid (EACA) is also described below.

(b) Alteration of the buffer systems for fractionation

Preliminary experiments were carried out using the fractionation procedure described by Cox *et al* (1973a). During the course of the present work, the buffer system used in this procedure was altered (Cox *et al*, 1978b). The revised basic procedure is outlined in Scheme IV.1., and described in full in Chapter II.H. The disintegration of cells was carried out as before (Cox *et al*,

SCHEME IV.1. FLOW DIAGRAM FOR THE FRACTIONATION OF MEMBRANES AND SOLUBILIZATION OF Mg-ATPase IN THE ABSENCE OF PAB



1973a) except that the protease inhibitor EACA was present at a concentration of 40mM in the STEM buffer system (see Chapter II.G.). For the wash at intermediate ionic strength ('50mM wash') and the washes at low-ionic strength ('5mM washes'), the Tris-HCl buffer system (Cox *et al*, 1973a) was replaced by a Tes-NaOH buffer system. The 50mM- and 5mM-Tes buffers also included 15%(v/v) glycerol and 40mM-EACA. The EDTA concentration (0.5mM) of the 5mM-Tes buffer was twice that of the 5mM-Tris buffer.

No investigation was made into any specific effects of the alteration of the buffer system or the inclusion of glycerol, although glycerol has been shown previously to stabilize the Mg-ATPase from *E.coli* (Kobayashi and Anraku, 1972; Vogel and Steinhart, 1976). However, if EACA was omitted from the whole procedure of Scheme IV.1, 70% of the Mg-ATPase activity present in the pooled low-ionic strength washes from strain AN248 (unc^+) was lost during concentration of the protein by ultrafiltration. No activity was lost when 40mM-EACA has been present throughout (Cox *et al*, 1978b).

(c) Fractionation in the absence of PAB

The revised procedure for the fractionation of membranes and concomitant solubilization of the Mg-ATPase is outlined in Scheme IV.1. Disintegrated cells were centrifuged at low speed to remove cell debris, and the supernatant was centrifuged at high speed to separate the 'cytoplasm' from the 'unwashed membranes'. The membranes are washed at intermediate ionic strength ('50mM-Tes wash'). The resulting membrane pellet is washed three times in

low-ionic strength (5mM-Tes) buffer, yielding a final membrane pellet ('EACA-washed membranes') and three supernatants ('EACA-washes') which are pooled and concentrated by ultrafiltration, to give the 'concentrated EACA-wash'. The concentrated EACA-wash contains about 60% of the Mg-ATPase activity originally present on the membranes (Cox *et al*, 1978b).

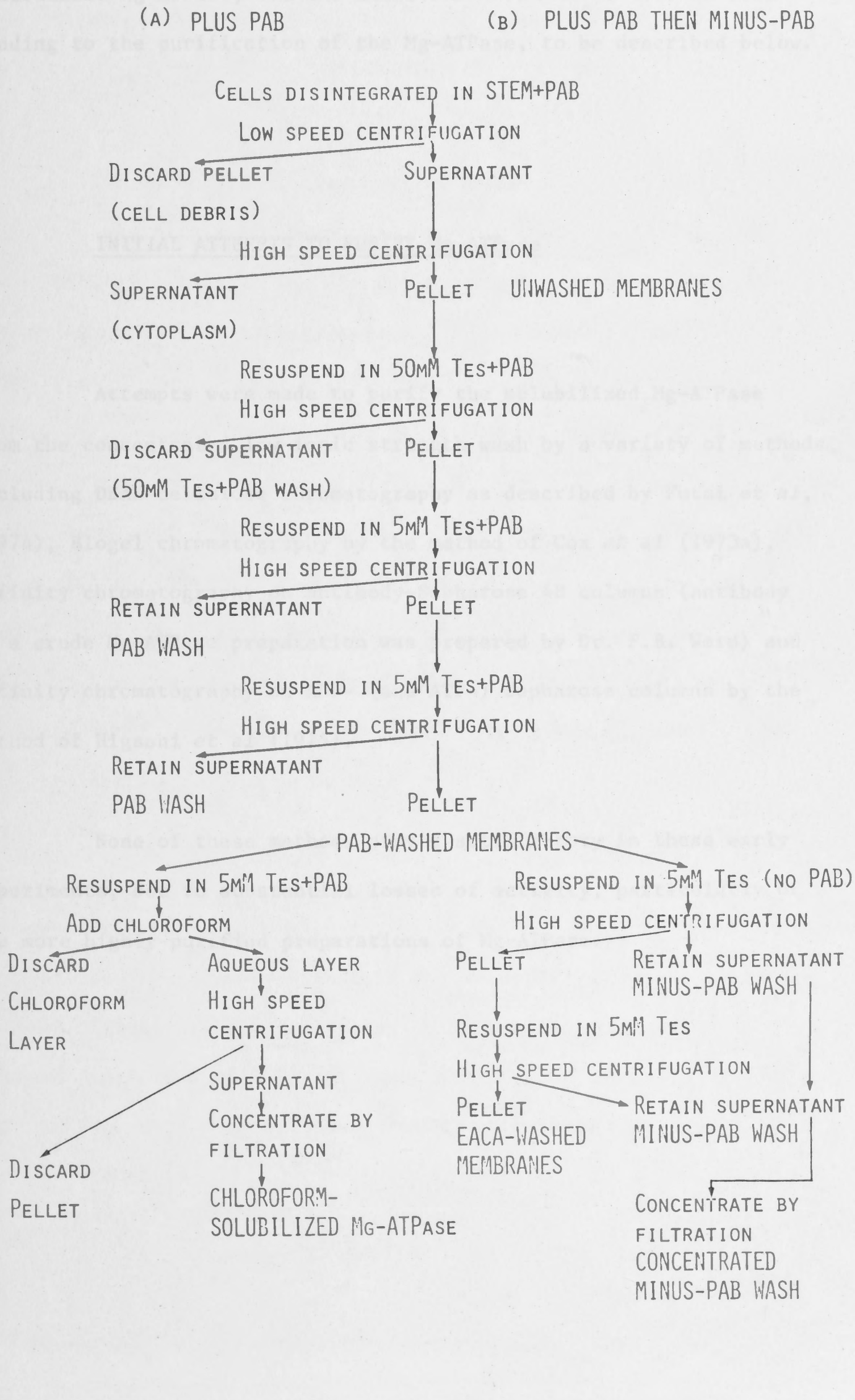
(d) Fractionation in the presence of PAB

As indicated above, the presence of PAB in all buffers during the fractionation procedure (Scheme IV.2a) completely prevented the solubilization of Mg-ATPase activity from membranes of strain AN248 (*unc*$^+$). The Mg-ATPase activity could be released by extraction of the 'PAB-washed membranes' with chloroform, which released the complex into the aqueous phase (Cox *et al*, 1978b). The method used was pioneered by Beechey *et al* (1975) for the solubilization of Mg-ATPase from beef heart mitochondrial membranes. The procedure is outlined in Scheme IV.2a. The aqueous fraction finally obtained is termed the 'chloroform-solubilized Mg-ATPase'.

(e) Solubilization of Mg-ATPase following the removal of PAB

If, after fractionation in the presence of PAB, the membranes from strain AN248 (*unc*$^+$) were washed twice at low-ionic strength in the absence of PAB, the Mg-ATPase activity was found in the soluble fraction ('Minus-PAB washes'). The membrane residue was similar to the stripped membranes obtained in the complete absence of PAB. Both preparations were termed 'EACA-washed membranes'. The 'Minus-PAB' washing procedure is outlined in Scheme IV.2b.

SCHEME IV.2. FLOW DIAGRAM FOR THE FRACTIONATION OF MEMBRANES IN THE PRESENCE OF PAB, FOLLOWED BY SOLUBILIZATION OF THE Mg-ATPase



Four preparations of solubilized Mg-ATPase - the low-ionic strength wash (Cox *et al*, 1973a), the EACA-wash, the chloroform-solubilized Mg-ATPase, and the Minus-PAB wash - were used in work leading to the purification of the Mg-ATPase, to be described below.

C. INITIAL ATTEMPTS TO PURIFY Mg-ATPase

Attempts were made to purify the solubilized Mg-ATPase from the concentrated low-ionic strength wash by a variety of methods, including DEAE-cellulose chromatography as described by Futai *et al*, (1974), Biogel chromatography by the method of Cox *et al* (1973a), affinity chromatography on antibody-Sepharose 4B columns (antibody to a crude Mg-ATPase preparation was prepared by Dr. F.B. Ward) and affinity chromatography on ADP- (and ATP-) Sepharose columns by the method of Higashi *et al* (1975).

None of these methods proved satisfactory in these early experiments, due to substantial losses of activity, particularly of the more highly-purified preparations of Mg-ATPase.

D. DEVELOPMENT OF A PROCEDURE FOR PURIFICATION OF Mg-ATPase BY TWO-DIMENSIONAL ELECTROPHORESIS

(a) Background

The method of electrophoresis under non-dissociating conditions was originally developed by Davis and Ornstein (Davis, 1964; Ornstein, 1964). The discontinuous gel contained: in the separating gel; 7%(w/v) acrylamide, 0.18%N,N'-methylenebisacrylamide, 0.38M-Tris-HCl buffer (pH8.8), 0.029% Temed, and 0.07% ammonium persulphate; and in the stacking gel 2.5%(w/v) acrylamide, 0.63% N,N'-methylenebisacrylamide, 62mM-Tris-HCl (pH 6.8), 20%(w/v) sucrose, 0.058% Temed, and 0.0005% riboflavin. The separating gel was allowed to polymerize, then the stacking gel was added, and the gel was exposed to strong fluorescent light for 30 min. During this exposure, the stacking gel polymerized. The electrode buffer contained 5mM-Tris and 38.4mM-glycine. After electrophoresis, the Mg-ATPase activity could be detected as a white lead phosphate precipitate in the gel following the ATP/lead staining procedure described in Chapter II. K(b). This staining procedure was based originally on a histological technique for the detection of phosphatases developed by Wachstein and Meisel (1957) from the method of Gomori (1939). The histological technique was adapted by Weinbaum and Markman (1966) for the staining of ATPase activity in gels, and the method used in the present work was a simplification of their procedure.

(b) Alterations made to the existing electrophoresis system

During the course of this work, the alterations described below were made to the Davis/Ornstein gel electrophoresis system.

(i) Increase in sample capacity

Initially, the sample capacity was increased by using slabs instead of cylindrical gels. Later, the thickness of the gel slab was increased from 1mm to 3mm, to accommodate much larger sample volumes and thus allow the purification of substantially greater amounts of Mg-ATPase on a single gel.

(ii) The inclusion of Mg^{2+}

Using the slab gel electrophoresis procedure, bands of Mg-ATPase activity were often very uneven. The inclusion of 1mM-ATP throughout the gel had no effect. Treatment of the samples with DNAase was equally ineffective. However, when 2mM-$MgSO_4$ was included throughout the gel and the electrode buffer (Abrams *et al*, 1976), the bands formed by the Mg-ATPase activity were straighter and more compact.

(iii) Polymerization of the stacking gel using persulphate

Since the existing method of polymerization of the stacking gel using riboflavin (Davis, 1964) produced a very sloppy gel (see also Righetti and Drysdale, 1976, p.443), and was also inconvenient to use, riboflavin was replaced by ammonium persulphate. The resulting gel was still very fragile and could not be handled, but it appeared to set more firmly, and was not as prone to breakage during the removal of well moulds.

(iv) Use of acrylamide gradients

The use of a gradient of acrylamide in the separating gel gave improved resolution of all bands, since bands did not broaden appreciably during electrophoresis. A summary and subjective evaluation of all gradients used is given in section (d) below.

(v) Stabilization of the acrylamide gradient with a glycerol gradient

To stabilize the acrylamide gradient during pouring and polymerization of the gel mixture, a gradient of glycerol was also included. Glycerol had been used by O'Farrell (1975) to stabilize acrylamide gradients. Also glycerol was known to have a protective effect on the Mg-ATPase (Kobayashi and Anraku, 1972; Davies and Bragg, 1972; Vogel and Steinhart, 1976). The sucrose in the stacking gel was also replaced by glycerol.

(c) Uses of the single-dimensional slab gel electrophoresis technique

The Mg-ATPase complexes could be purified or partially purified from any fraction containing solubilized Mg-ATPase activity using the single-dimensional electrophoresis system. Inactive Mg-ATPase complexes, such as that from strain AN249 (*uncA401*) could also be partially purified using this system. An active Mg-ATPase preparation and the inactive preparation were placed in adjacent wells. The inactive complex was found to have the same mobility as the active band.

(d) Isoelectric focusing under non-dissociating conditions

(i) Initial work

Since isoelectric focusing was a powerful technique for resolving proteins from complex mixtures, the possibility was investigated that isoelectric focusing could separate the Mg-ATPase complex from other proteins in crude solubilized preparations.

For the initial experiments, gels contained 18-26%(v/v) glycerol (or sucrose at comparable densities), and 3.5%, 4% or 10%(w/v) total acrylamide (T), with appropriate amounts of catalysts. The acrylamide solution used was solution D of O'Farrell (1975) (see Chapter II.M(a)) which contains N,N'-methylenebisacrylamide to give 5.7% cross-linking (C). Cylindrical gels (T=4%, C=5.7%) containing 25% glycerol were selected as the most suitable for subsequent experiments. Glycerol was chosen because of its stabilizing effect on Mg-ATPase (Kobayashi and Anraku, 1972; Vogel and Steinhart, 1976).

(ii) Development of a method for isoelectric focusing of Mg-ATPase

It was found that the Mg-ATPase did not enter gels containing Ampholines of the composition 1:1:1:2 (pH ranges 3.5 to 5: 5 to 7: 7 to 9: 9 to 11), but did focus in gels containing 1:1:1 (pH ranges 3.5 to 5: 5 to 7: 7 to 9). In the former case, the cathode buffer was 0.2M-NaOH, and in the latter, a solution of Ampholines was used (0.8% (pH 9 to 11) and 0.2% (pH 7 to 9)). In both cases the anode solution was 0.1M-H_3PO_4, the sample was applied at the cathode end and gels were run for 6 hours at a current of approximately 1mA per gel, maintained by frequent voltage increases.

These experiments indicated that the Mg-ATPase focused in a broad band between pH 5 and 6. A number of pH ranges covering the neutral to acidic region were tested. The most satisfactory focusing was obtained using the Ampholine composition of 3:2 (pH ranges 4 to 6: 6 to 8), with 10mM-H_3PO_4 and the alkaline Ampholine solution as electrode buffers. A further improvement was obtained when 0.2% ethanolamine was used as the cathode buffer as described by Emes *et al* (1975). The duration of isoelectric focusing was also increased to 9-11 hours.

The final procedure adopted for isoelectric focusing of the Mg-ATPase is described in Chapter II.K(a). The pH gradient formed is shown in Fig. IV.1.

(e) Two-dimensional electrophoresis under non-dissociating conditions

The isoelectric focusing gels described in section (c) and the slab gel electrophoresis system described in section (b), were combined into a two-dimensional electrophoresis procedure as described below.

(i) Sealing the isoelectric focusing gel to the slab gel

An adequate sealing gel was obtained by melting 1.5%(w/v) agarose in a small amount of the electrode buffer used in the second dimension. The untreated isoelectric focusing gel was mounted above the stacking gel of the slab electrophoresis system, and the agarose gel (cooled to about 40°C) was poured to just cover the cylindrical gel.

Considerable savings in effort and materials could be

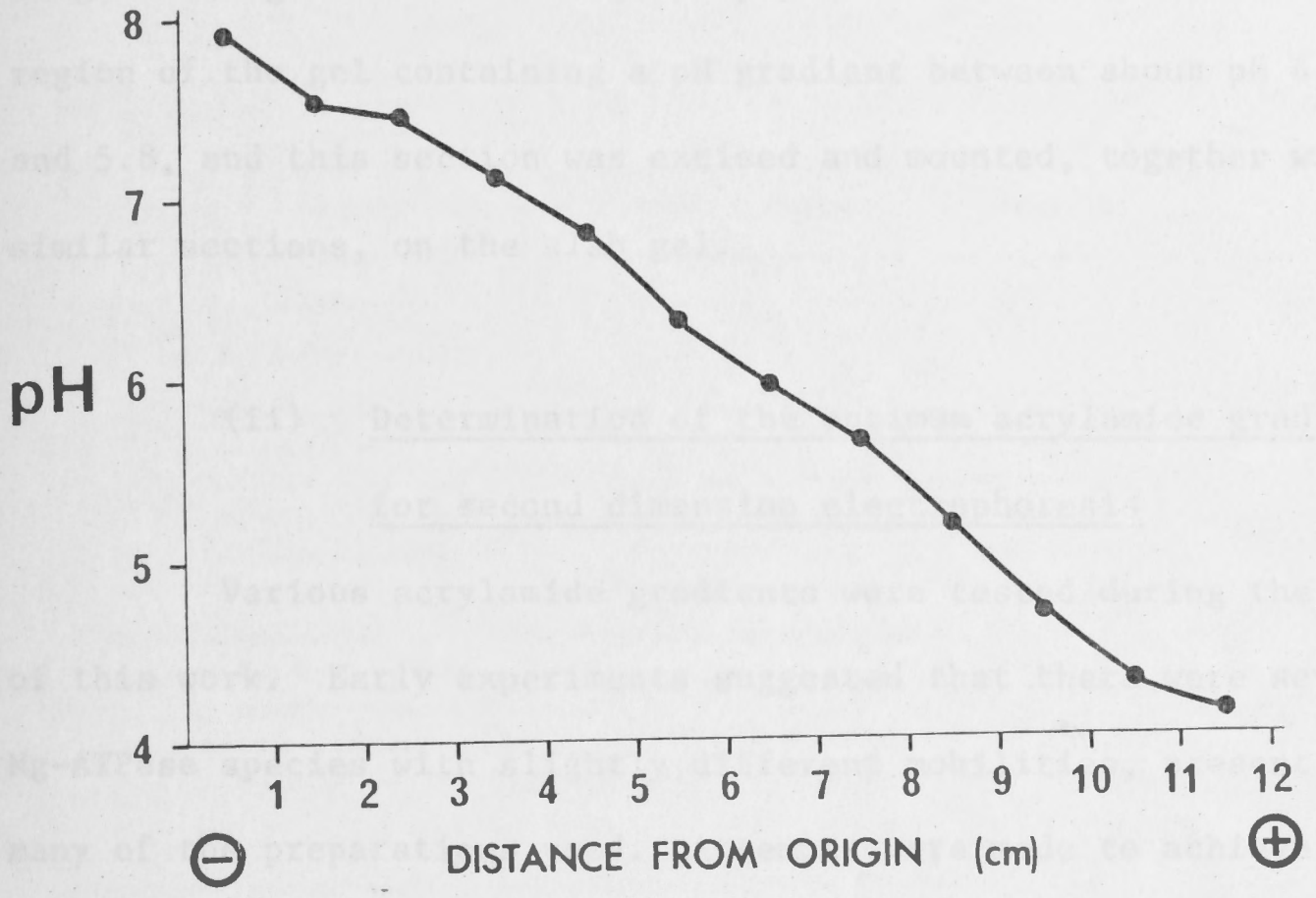


Figure IV.1. The pH gradient of the isoelectric focusing gel used in the purification of Mg-ATPase complexes. The Ampholine composition was 3:2 (pH ranges 4 to 6: 6 to 8). Segments (5mm thick) were incubated in 2.5ml of water, and the pH was measured. Gels were run for a total of 9 hours, at constant current to give 180V initially, increasing to a maximum of about 500V, which was maintained for the rest of the run.

achieved by mounting up to four isoelectric focusing gels on a single slab gel. Narrow range pH papers were used to isolate the region of the gel containing a pH gradient between about pH 4.8 and 5.8, and this section was excised and mounted, together with similar sections, on the slab gel.

(ii) Determination of the optimum acrylamide gradient for second dimension electrophoresis

Various acrylamide gradients were tested during the course of this work. Early experiments suggested that there were several Mg-ATPase species with slightly different mobilities, present in many of the preparations used. Attempts were made to achieve optimum resolution of such species, and the acrylamide compositions and gradients used are listed in Table IV.1, together with a subjective evaluation of the separations obtained. The identities of the various species of Mg-ATPase complex will be discussed in Chapter V.

A gel slab of the composition T=6 to 7.5%, C=5.7% (Table IV.1) was eventually chosen, since this gradient gave the most consistent and effective resolution of the various types of Mg-ATPase. The stacking gel (T=3.13%, C=20%; Davis, 1964) proved necessary for optimal resolution (Table IV.1). Gels containing higher degrees of cross-linking gave particularly good resolution, but the gels tended to be opalescent, and it was hard to distinguish the various bands or spots of Mg-ATPase activity during the early stages of staining. Short staining times were desirable in order to minimize both loss of protein from the gels, and also the salt content of the active region, since high ionic strength interferes with the subsequent analytical isoelectric focusing.

Table IV.1 A subjective evaluation of the resolution obtained with the various acrylamide gradients used for slab gel electrophoresis under non-dissociating conditions. Four categories of separation between various species of Mg-ATPase (which are described in Chapter V) were established for the assessments of the resolution of a gel:

(a) Resolution of the major and minor bands found in gels overloaded with normal Mg-ATPase solubilized by low-ionic strength treatment.

(b) Resolution of two major species of normal Mg-ATPase solubilized by chloroform treatment.

(c) Resolution of the mobility difference between the normal Mg-ATPases solubilized by low-ionic strength or chloroform treatments.

(d) Partial division of the major spot formed by normal Mg-ATPase solubilized by low-ionic strength treatment.

Subjective assessments # of the degree of resolution have been classified as follows:

- \- little or no separation
- ± variable but low separation
- \+ some separation
- ++ separation such that the species could be excised separately using a razor blade.

Degree of Cross- linking (C)	Total Acrylamide Concentration (T) Top to Bottom	Current (mA)	Time (hrs)	Categories of Separation (a)	(b)	(c)	(d)
WITHOUT STACKING GEL							
5.7%	2.4 - 8.5%	22	12	-	-	±	-
WITH STACKING GEL							
2.6%	2.9 - 12.2%	20	13.5	-			-
	5.7 - 10.1%	20	10	-			-
	5.7 - 8.6%	18	10.3	-			-
		20	12	±	+	++	-
5.7%	5.25 - 6.75%	20	12	++	++	++	±
	5.6 - 6.4%	20	12	+	+	-	-
	6 - 9%	20	12		++		
	6 - 7.5%	20	12	++	++	-	±
		22	12	++	++	++	+
	*	20	16	++	++	++	+
	6 - 6.75%	20	12	-			-
	6.75 - 8.25%	20	12	-	+	-	-
	6.75 - 7.5%	20	12	++	++	-	-
8.6%	6 - 6.75%	20	12	++			±
11.4%	6 - 7.5%	20	12	++	++	+	±
	6 - 6.75%	20	12	++	++	++	±
11.4 - 2.6% (top to bottom)	6.75%	20	16	+	+	+	+

* This gradient and electrophoresis schedule was adopted.

\# Any quantitative estimation of the degree of separation between species is difficult, since the sharpness and width of each band varies with such factors as the sample load, the distance travelled in the gel, and the length of staining time. A subjective evaluation has therefore been made, based on the criteria of separate excision, partial separation (judged visually), and reproduc ibility of such separation.

At a given total acrylamide concentration (T), the mean pore-size is known to decrease with decreasing percentage cross-linking (see Chrambach and Rodbard, 1971 ; and Righetti and Drysdale, 1976). However, a gel containing a gradient of cross-linker (T=6.75%, C=11.4 - 2.6%, Table IV.1) gave no improvement in resolution under the conditions tried.

(iii) The electrophoresis conditions for the second dimension

Electrophoresis at currents above 22mA generated considerable heat, and it was decided to extend the electrophoresis time at 20mA from 12 h. to 16 h. to give a marginal improvement in resolution (Table IV.1; T=6 - 7.5%, C=5.7%). During such a procedure, the bottom section (about 1cm) of the slab gel tended to disintegrate.

(f) The recommended method

The procedure for two-dimensional electrophoresis of intact Mg-ATPase which gave the best results is outlined below. This method is described in full in Chapter II.K. Samples of the Minus-PAB wash are placed on cylindrical gels (T=4%, C=5.7%) containing 25% glycerol and Ampholines of the composition 3:2 (pH ranges 4 to 6: 6 to 8). The cathode and anode solutions are 0.2% ethanolamine and 10mM-H_3PO_4 respectively. Isoelectric focusing is performed for a total of 9 hours, at a constant current to give initially 180V, rising to about 500V, and at a constant 500V for the remainder of the run. Second dimension electrophoresis is carried out using a 3mm-thick discontinuous slab gel. The separating gel contains a gradient of

acrylamide (T=6 to 7.5%, C=5.7%) stabilized by a glycerol gradient. The stacking gel (T=3.13%, C=20%), which is polymerized using ammonium persulphate, also includes glycerol. 2mM-$MgSO_4$ is present throughout the gel and the electrode buffer. Gels are run at 20mA for 16 hours.

E. DISCUSSION

The protease inhibitor PAB had been found to prevent the solubilization of the Mg-ATPase which normally occurred during the repeated low-ionic strength washing of membranes. It was shown that when PAB was removed during this washing procedure, the Mg-ATPase activity was found in the soluble fraction. These observations were utilized to obtain a preparation of Mg-ATPase which had high specific activity (see Chapter V) when compared with preparations obtained by low-ionic strength washing in the absence of PAB.

A second method of solubilizing the Mg-ATPase was by chloroform extraction of the PAB-washed membranes. The resulting preparation contained Mg-ATPase activity, but could not reconstitute energy-linked activities in stripped membranes. This suggested that the complex solubilized by chloroform treatment was different to that solubilized by low-ionic strength treatment, and the nature of this difference will be discussed in the following chapter.

As discussed in Chapter I, the Mg-ATPases isolated from a number of organisms are cold labile. In the present work, the early

experiments involving purification of Mg-ATPase from the low-ionic strength wash were plagued by extensive losses of activity, particularly during the later stages of purification. All these early experiments were carried out in the absence of EACA and glycerol. Kobayashi and Anraku (1972) and Davies and Bragg (1972) had shown that glycerol protected the Mg-ATPase against inactivation at 0^{o} to $4^{o}C$. Subsequently, Vogel and Steinhart (1976) showed that glycerol protected the enzyme at low temperatures from a reversible loss of Mg-ATPase activity.

In the present work, the soluble Mg-ATPase activity of the EACA- or Minus-PAB-washes was not cold labile, and was retained after freezing and thawing. In addition, the presence of the protease inhibitor EACA was shown to protect Mg-ATPase activity during ultrafiltration at $4^{o}C$ in the presence of glycerol, and it is possible that EACA prevents inactivation of the Mg-ATPase under other conditions as well. Thus it is likely that the loss of activity experienced during early work could have been largely prevented by the use of the revised fractionation procedure.

A two-dimensional electrophoresis procedure for the purification of solubilized Mg-ATPase was developed when other purification methods had proved unsatisfactory. Improvements were made to the two-dimensional procedure with the aim of investigating possible differences between the chloroform-solubilized Mg-ATPase and the Mg-ATPase found in the Minus-PAB wash. The results of such a study are described in Chapter V.

During the refinement of the second dimension of the technique, many of the gradients were tested using inappropriate electrophoresis times and currents, and it is likely that resolution could be improved by further variation of these parameters. Moreover, it is possible that good resolution could be obtained using a suitable single acrylamide concentration, as suggested by the theoretical work of Rodbard *et al* (1971). The many factors contributing to the resolution by pore gradient gel electrophoresis have been analysed and discussed by Rodbard *et al* (1971) and Chrambach and Rodbard (1971). The role of the 'pore' has recently been questioned by Bode (1977).

Following the development of the purification method, a search of the literature revealed a number of existing procedures for two-dimensional electrophoresis under non-dissociating conditions using isoelectric focusing in the first dimension (Dale and Latner, 1969; Macko and Stegemann, 1969; Kenrick and Margolis, 1970; Wright *et al*, 1973; Emes *et al*, 1975). Several of these methods also employ pore gradient electrophoresis in the second dimension (Kenrick and Margolis, 1970; Wright *et al*, 1973; Emes *et al*, 1975, 1976).

Chapter V

CHARACTERIZATION OF THE Mg-ATPases SOLUBILIZED BY LOW-IONIC STRENGTH OR CHLOROFORM TREATMENTS FROM THE CYTOPLASMIC MEMBRANE OF STRAIN AN248 (*unc*$^+$)

A. INTRODUCTION

In the preceding chapter, various methods were described by which Mg-ATPase activity could be solubilized from membranes of normal strains. These methods for the solubilization of Mg-ATPase involved either the low-ionic strength washing of membranes, or the extraction of membranes with chloroform. The former method produces Mg-ATPase capable of binding to normal membranes stripped of Mg-ATPase, to reconstitute ATP-driven energy-linked processes (Bragg and Hou, 1972; Cox *et al*, 1973a, 1978a). The chloroform-solubilized Mg-ATPase, however, cannot reconstitute ATP-induced membrane energization in EACA-washed membranes, as judged by the lack of ATP-dependent atebrin fluorescence quenching in the reconstituted membranes (Cox *et al*, 1978b). Moreover, the chloroform-solubilized Mg-ATPase cannot repair the proton 'leakiness' characteristic of membranes stripped by the EACA-washing procedure, as judged by the lack of NADH-induced atebrin fluorescence quenching in the reconstituted membranes. In contrast, the binding of the low-ionic strength-solubilized Mg-ATPase restores the low proton-permeability to such membranes (Cox *et al*, 1978b).

Since the Mg-ATPase solubilized by low-ionic strength treatment was active in reconstitution, but the chloroform-solubilized

Mg-ATPase was not, the structures of the two complexes were presumably different. The first part of this chapter describes the use of the electrophoretic purification procedures described in Chapter IV, together with the analytical procedures outlined in Chapter III, to characterize the Mg-ATPase complexes solubilized from membranes of strain AN248 (*unc*$^+$).

B. ANALYSIS OF CRUDE Mg-ATPase PREPARATIONS FROM STRAIN AN248 (*unc*$^+$) BY GEL ELECTROPHORESIS

The preparations containing solubilized Mg-ATPase activity were the low-ionic strength wash, the EACA-wash, the Minus-PAB wash, and the chloroform-solubilized Mg-ATPase (see Chapter IV). The analysis of the composition of these crude preparations is described in the following sections.

(a) Electrophoresis under non-dissociating conditions

The Mg-ATPase complexes found in the concentrated low-ionic strength wash (Fig.V.1a) and the chloroform-solubilized preparation (Fig.V.1b) had similar mobilities on gels under non-dissociating conditions. However, the gel used in Fig.V.1 gave poor resolution since it contained a steep acrylamide gradient (T = 2.4 to 8.5%) with no stacking gel (see Table IV.1). Gels giving better resolution showed that the chloroform-solubilized Mg-ATPase (Fig.V.1f) had marginally higher mobility than the low-ionic strength solubilized Mg-ATPase (Fig.V.1e). (In Table IV.1, category (C) refers to the

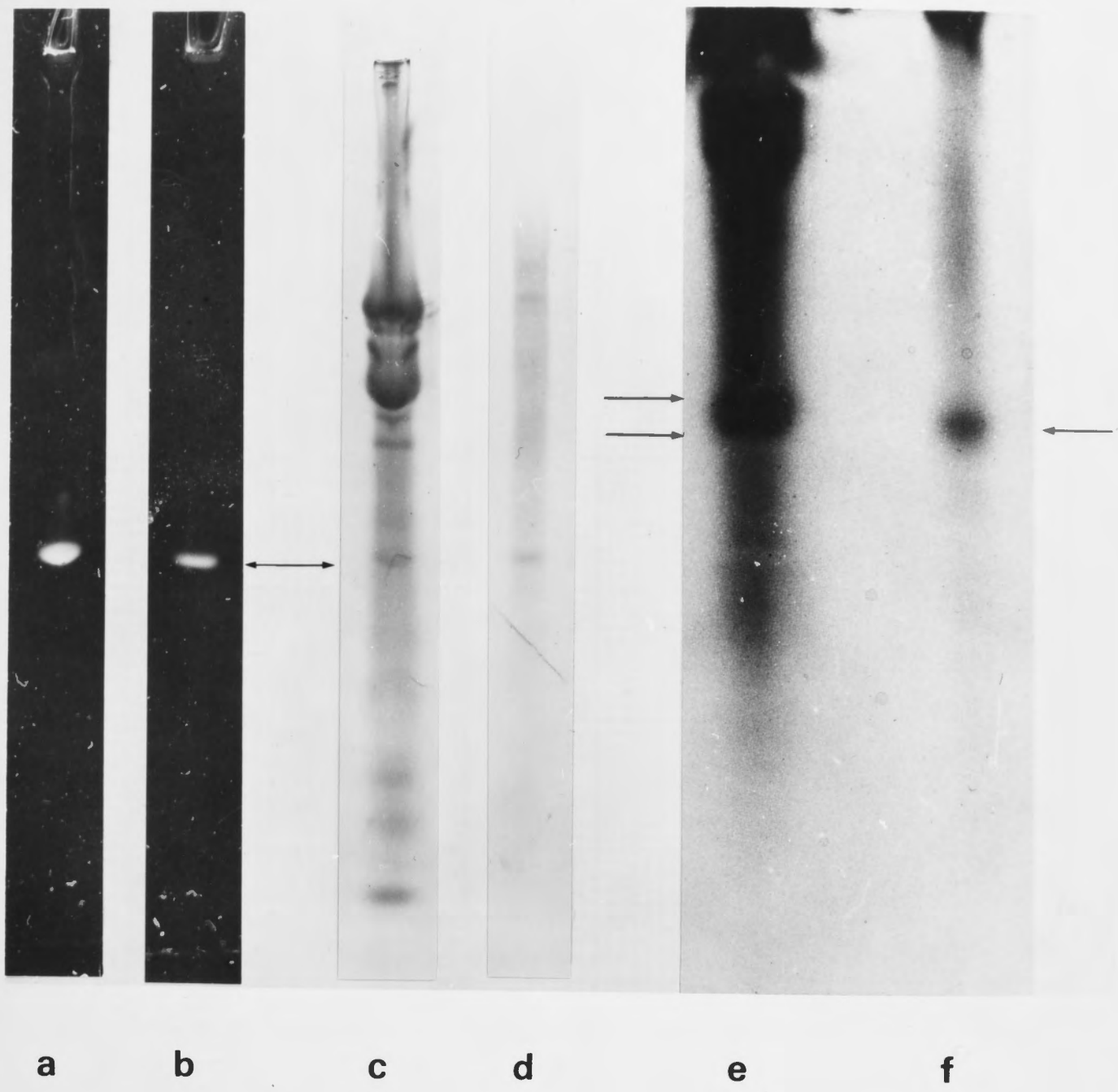


Figure V.1. Electrophoresis under non-dissociating conditions of the concentrated low-ionic strength wash (a,c,e), and the chloroform-solubilized Mg-ATPase (b,d,f) preparations from strain AN248 (*unc*$^{+}$). One gel containing the concentrated low-ionic strength wash (530µg of protein) was stained for activity (a) and then for protein (c). An adjacent well containing the chloroform-solubilized Mg-ATPase (40µg of protein was stained for activity (b) and then for protein (d). Non-dissociating electrophoresis (a-d) was performed for 12 hours at 22mA in a gel containing a gradient of 2.4% to 8.5%(w/v) acrylamide, with no stacking gel. The same preparations were run for 12 hours at 20mA using a gradient of 5.25 to 6.75%(w/v) acrylamide; (e) concentrated low-ionic strength wash (530µg of protein); (f) chloroform-solubilized Mg-ATPase (48µg of protein). One gel (a-d) was stained for Mg-ATPase activity, and photographed (a,b) through a polarizing filter, using a diffuse light source polarized at 90^{o} to the filter. Both gels (c,d and e,f) were stained for protein with Coomassie blue. The preparations used for samples (a-f) were concentrated through a Diaflo XM-50 ultrafilter. Other details of the methods are given in Chapter II.

resolution of the mobility difference between these bands).

The specific Mg-ATPase activity of the concentrated low-ionic strength wash (Fig.V.1a) was 0.5 units/mg of protein, compared to 15 units/mg of protein for the chloroform-solubilized Mg-ATPase (Fig.V.1b) (Cox *et al*, 1978b). (One unit of activity = 1μmol P_i produced per min). The difference in specific activity is reflected in the protein profiles obtained when the gel which had been stained for activity (Fig.V.1a,b) was stained for protein (Fig.V.1c,d).

(b) Electrophoresis under dissociating conditions

The preparations shown in Fig.V.1a-d were also dissociated in SDS and mercaptoethanol at 100°C (see Chapter II.L) and subjected to one-dimensional SDS electrophoresis (Chapter II.M(c)). The profiles obtained, together with that of a similar gel of the Minus-PAB wash, are shown in Fig.V.2. The specific Mg-ATPase activities of the concentrated Minus-PAB wash (5.5 units/mg of protein) and the chloroform-solubilized Mg-ATPase (15 units/mg of protein) were reflected in the high concentrations of the Mg-ATPase subunits. In both the Minus-PAB wash (Fig.V.2c) and the chloroform-solubilized Mg-ATPase (Fig.V.2b) preparations, the major high molecular weight polypeptides were the α- and β-subunits of the Mg-ATPase. In contrast, these subunits formed a much less prominent part of the protein profile of the concentrated low-ionic strength wash (Fig.V.2a). The bands formed by each of the subunits (Fig.V.2) were identified as described in section D.

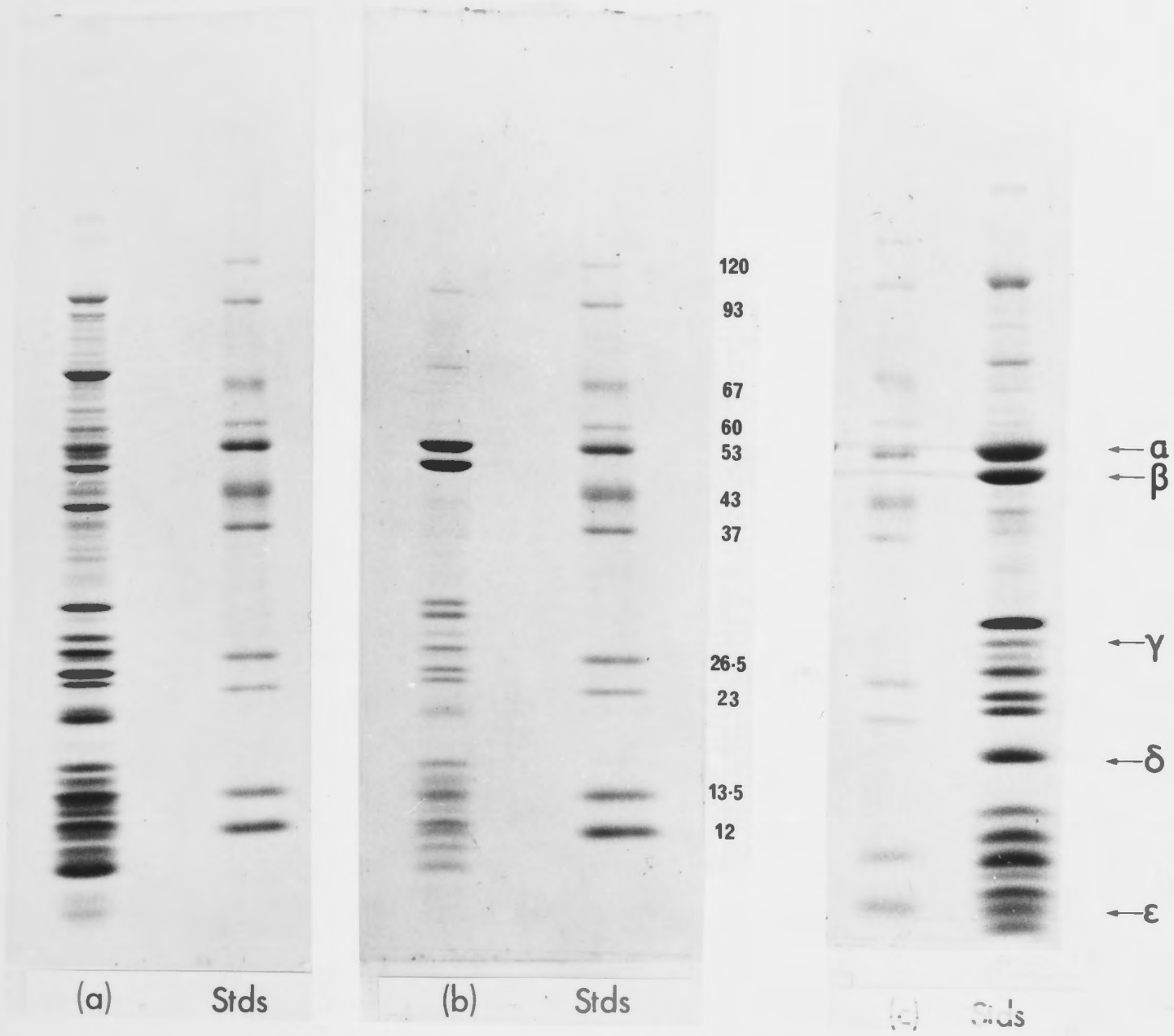


Figure V.2. SDS electrophoresis of the concentrated low ionic strength wash (a; 22µg of protein), the chloroform-solubilized Mg-ATPase (b; 9µg of protein), and the Minus-PAB wash (c; 30µg of protein) preparations from strain AN248 (*unc*$^{+}$). Gels were stained for protein with Coomassie blue. The positions of the five subunits of the Mg-ATPase present in the Minus-PAB wash (c) are indicated. Molecular weight standard proteins (Stds) were: β-galactosidase (120,000), phosphorylase A (93,000), bovine serum albumin (67,000), catalase (60,000), glutamate dehydrogenase (53,000), ovalbumin (43,000), alcohol dehydrogenase (37,000), triosephosphate isomerase (26,500), adenylate kinase (23,000), ribonuclease A (13,500), cytochrome c (12,000). Molecular weights ($x10^{-3}$) of the protein standards are indicated. Samples (a) and (b) were concentrated using a Diaflo XM-50 ultrafilter, and sample (c) with the PM-10 ultrafilter. All other conditions are as described in Chapter II.

C. PURIFICATION AND RESOLUTION OF Mg-ATPase COMPLEXES FROM STRAIN AN248 (*unc*$^+$) BY TWO-DIMENSIONAL ELECTROPHORESIS UNDER NON-DISSOCIATING CONDITIONS

In the preceding chapter, a method was described for the purification of Mg-ATPase in active form by two-dimensional electrophoresis. This section describes the use of the method for the purification of the Mg-ATPase from the EACA- or Minus-PAB washes, or the chloroform-solubilized preparation, from strain AN248 (*unc*$^+$).

During purification using this method, several different species of Mg-ATPase complex were detected and resolved.

(a) A comparison of the Mg-ATPases solubilized by low-ionic strength or by chloroform treatment: isoelectric points

As indicated in Chapter IV, preliminary experiments indicated that the Mg-ATPase had a pI between pH 5 and 6. However, the activity of the Mg-ATPase after focusing was apparently very low, as indicated by very slow staining by the lead phosphate staining procedure described in Chapter II.K(b). This may be because the enzyme has low activity at acidic pH (Evans, 1969). When this band of Mg-ATPase is electrophoresed into a slab gel as described below, the resulting spot stains rapidly. The slab gel is buffered at pH 8.8, close to the optimal pH for solubilized Mg-ATPase activity. (Davies and Bragg, 1972; Bragg and Hou, 1967).

Further experiments indicated that the chloroform-solubilized

Mg-ATPase tended to focus between pH 5.3 and 5.1, whilst the Mg-ATPase solubilized by low-ionic strength treatment formed a broad band of activity from pH 5.5 to 5.1. These bands were not appreciably sharpened by increasing the duration of electrofocusing, or by decreasing the protein loads.

(b) A comparison of the Mg-ATPases solubilized by low-ionic strength or by chloroform-treatment: molecular sizes

An indication of the difference in molecular weight between the complexes could be gained from their relative mobilities in acrylamide-gradient gels in comparison to the mobilities of proteins of known molecular weight. The results of such experiments are collated in Fig.V.3. Two active species could be distinguished in the chloroform-solubilized Mg-ATPase preparation, and their approximate molecular weights were 340,000 and 310,000, estimated by comparison with the standard proteins indicated (Fig.V.3). In comparison, the active species present in the Minus-PAB wash had a molecular weight of about 360,000. The limitations involved in such estimations have been discussed by Rodbard *et al* (1971).

(c) Two-dimensional electrophoresis under non-dissociating conditions of the Mg-ATPases solubilized by low-ionic strength or chloroform treatments

The solubilized Mg-ATPase complexes were purified from the low-ionic strength wash, the EACA-wash, the Minus-PAB wash, or the chloroform-solubilized preparation, by two-dimensional electrophoresis. The best results were obtained using samples of the concentrated

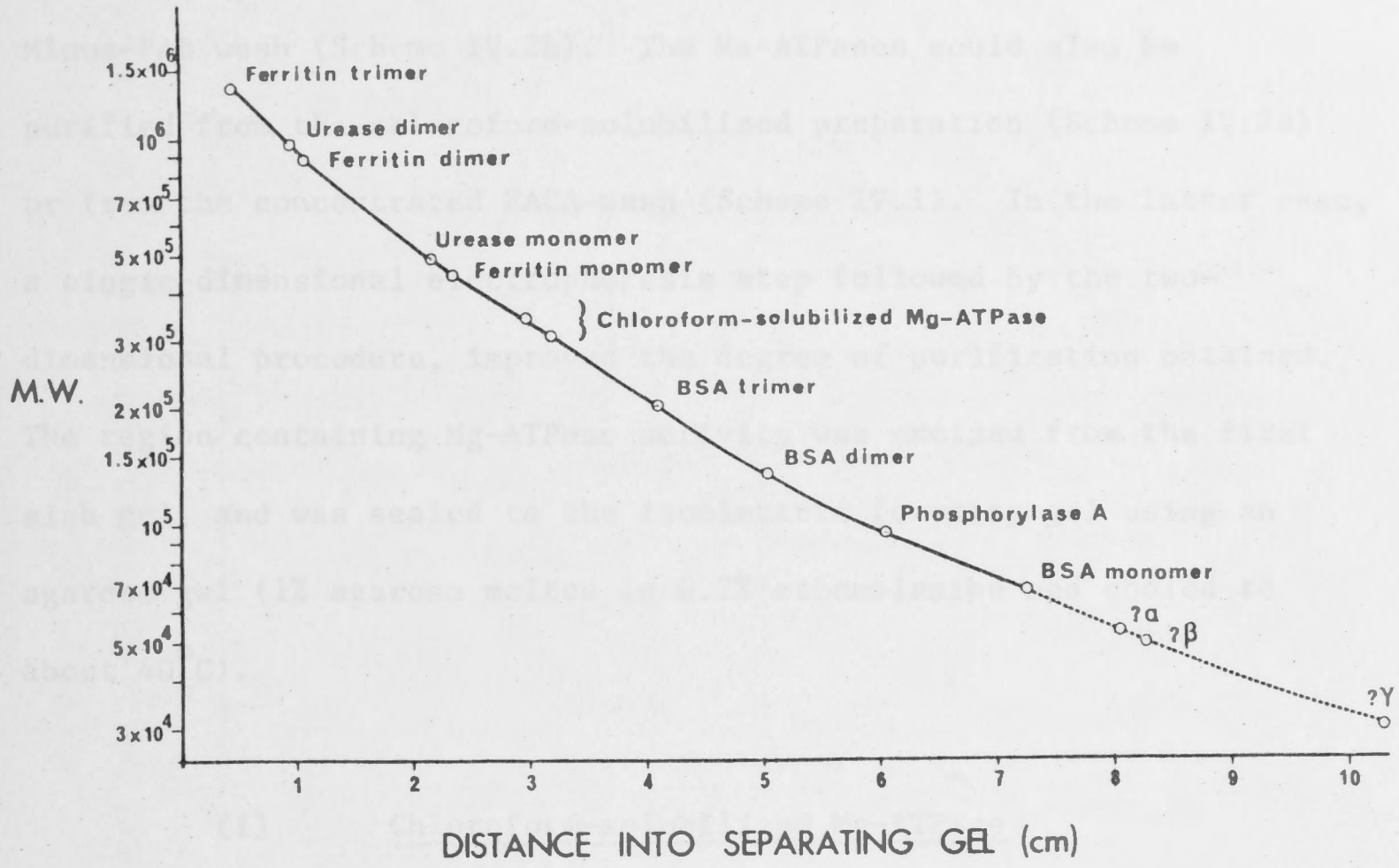


Figure V.3. Electrophoretic mobility of proteins on gels under non-dissociating conditions, as a function of the logarithm of molecular weight. The results from several separate experiments were collated. All distances were standardized to those on a gel (T=5.7-8.6%, C=2.6%; 20mA, 12 hours) on which urease and the chloroform-solubilized Mg-ATPase from AN248 (*unc*$^+$) were run (see also Fig. V.10). Data from other gels were corrected by multiplying the mobility of each band by a constant factor, (mobility of *x* in standard gel/mobility of *x* in sample gel) using any protein *x* present in both gels. On a second gel (T=5.7-8.6%, C=2.6%; 20mA, 12 hours) the positions of the bands of ferritin were compared with the urease bands. A third gel (T=6-7.5%, C=5.7%; 20mA, 12.25 hours) contained bovine serum albumin (BSA) and phosphorylase A, both solubilized with SDS, and a chloroform-solubilized Mg-ATPase preparation. The low molecular weight bands labelled '?α' and '?β' could be seen on this gel. The '?α' and '?β' bands, plus a third band labelled '?γ', could also be seen on the standard gel. Data from several other gels could be superimposed on this curve after correction. The molecular weight of urease was taken to be 485,000, and ferritin 440,000 (see Righetti and Caravaggio, 1976; and Darnall and Klotz, 1975). The molecular weights of SDS treated proteins are given in the legend to Fig. V.2.

Minus-PAB wash (Scheme IV.2b). The Mg-ATPases could also be purified from the chloroform-solubilized preparation (Scheme IV.2a) or from the concentrated EACA-wash (Scheme IV.1). In the latter case, a single-dimensional electrophoresis step followed by the two-dimensional procedure, improved the degree of purification obtained. The region containing Mg-ATPase activity was excised from the first slab gel, and was sealed to the isoelectric focusing gel using an agarose gel (1% agarose melted in 0.2% ethanolamine and cooled to about 40^{o}C).

(i) Chloroform-solubilized Mg-ATPase

Two-dimensional electrophoresis of chloroform-solubilized Mg-ATPase preparations from strain AN248 (*unc*$^{+}$) indicated that there were two active species present (Fig.V.4a). The two types of complex were separated in both dimensions, the complex of higher mobility having the more acidic apparent pI. This observation is consistent with the data from the single-dimensional gels (Fig.V.3). (In Table IV.1, category (b) refers to the separation between these two species).

(ii) Mg-ATPase from the Minus-PAB wash

The Mg-ATPase activity solubilized by Minus-PAB washing of membranes from strain AN248 (*unc*$^{+}$) showed a gel pattern (Fig.V.4c) different from that of the chloroform-solubilized Mg-ATPase (Fig.V.4a). One spot was formed by the Mg-ATPase from the Minus-PAB wash although occasionally this spot appeared to be splitting in the second-dimension (Fig.V.4c) or in single-dimensional slab gels, but

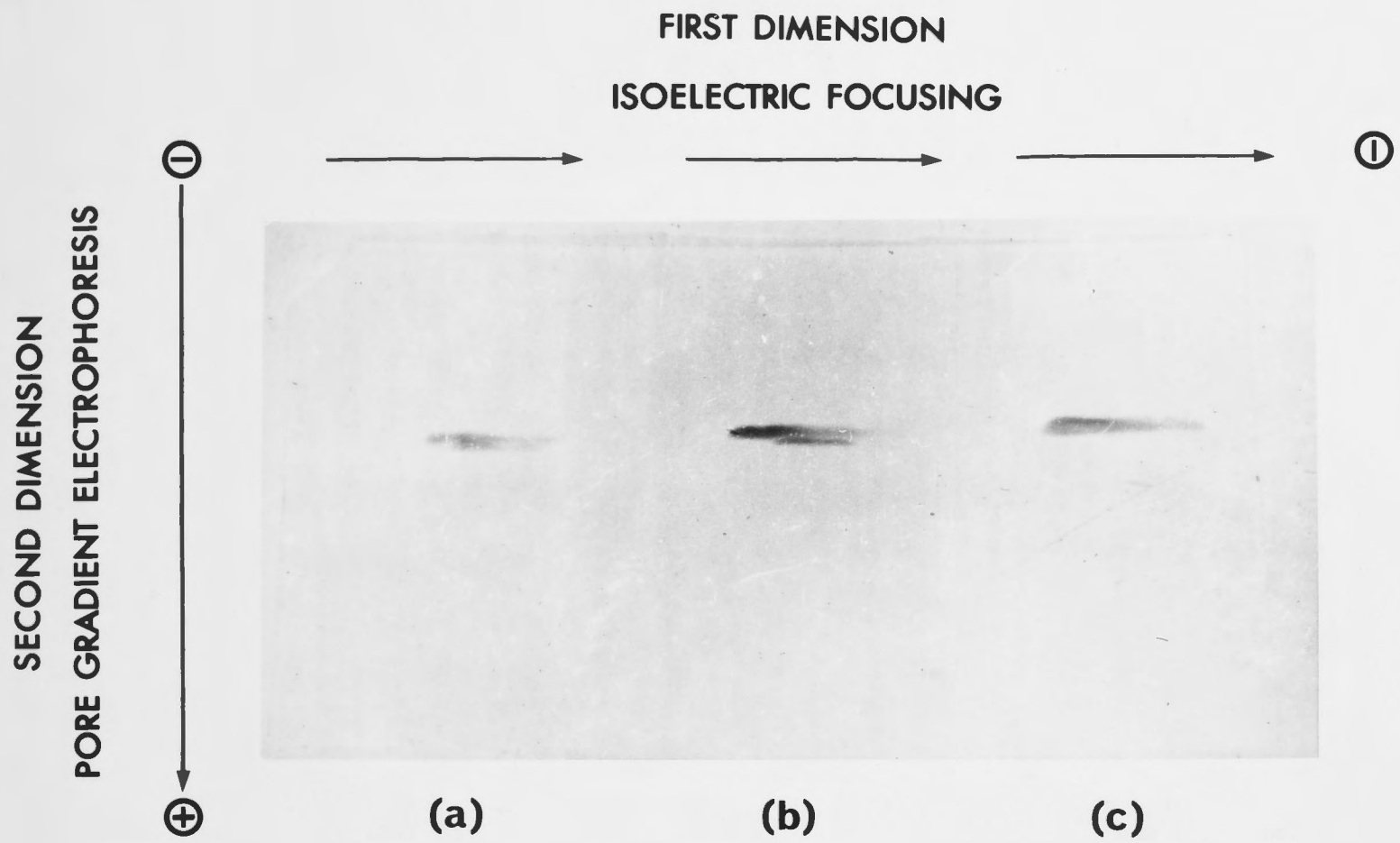


Figure V.4. Two-dimensional gel electrophoresis (non-dissociating conditions) of the chloroform-solubilized Mg-ATPase (a), the Minus-PAB wash (c), and a mixture of the two preparations (b), from strain AN248 (*unc*$^{+}$). Sample (a) contained 0.9mg of protein, sample (c) contained 1.3mg of protein, and sample (b) contained 0.7mg of protein of the chloroform-solubilized preparation plus 1mg of protein of the Minus-PAB wash. Each sample was subjected to electrophoresis in the direction indicated. Sections from the three isoelectric focusing gels were mounted on a single slab for electrophoresis in the second dimension. The gels were stained for Mg-ATPase activity and photographed between filters of high neutral density (zero polarization). The bottom centimetre of the slab gel disintegrated partially during electrophoresis, and was removed before the gel was processed. The conditions for electrophoresis were as described in Chapter II.K.

the fine structure of the spot or band was not resolved. (In Table IV.1, category (d) refers to the detection of the division of this spot).

The Mg-ATPase from the EACA-wash exhibited a similar pattern, although there were also species present which stained much more slowly than the Mg-ATPase. These spots were probably formed by contaminating cytoplasmic proteins, such as the pyrophosphatase (M.W. 118,000) described by Wong *et al* (1970).

(iii) Co-electrophoresis

When the chloroform-solubilized Mg-ATPase and the concentrated Minus-PAB wash were subjected to electrophoresis together, the pattern shown in Fig.V.4b was obtained. The spot with the higher mobility in the second-dimension clearly corresponds with the high mobility ('310,000') spot of the chloroform-solubilized Mg-ATPase pattern (Fig.V.4a). The fine structure of the major spot is not resolved; however it is apparent that part of the Mg-ATPase activity is focusing at a more alkaline isoelectric point than the low mobility ('340,000') spot of the chloroform-solubilized Mg-ATPase profile. The extra area of Mg-ATPase activity is presumably due to the complex from the Minus-PAB wash.

When gels were overloaded with the EACA-or Minus-PAB washes, an additional minor spot was seen at a higher mobility and more acidic isoelectric point than the major spot (results not shown). The amount of protein in the minor spot was very small. (In Table IV.1, category (a) refers to the separation between the major and minor species).

Electrophoresis of the chloroform-solubilized Mg-ATPase together with large amounts of the Minus-PAB wash (gels not shown) revealed that the minor spot seen in the latter preparation ran in the same position as the high-mobility ('310,000') species of the chloroform-solubilized preparation.

D. ANALYSIS OF THE SUBUNITS OF THE Mg-ATPase COMPLEX PURIFIED FROM THE MINUS-PAB WASH OF STRAIN AN248 (*unc*$^{+}$)

(a) Polypeptide composition of the Mg-ATPase purified from the Minus-PAB wash

The polypeptide composition of the Mg-ATPase complex solubilized by Minus-PAB washing and purified by two-dimensional electrophoresis, is shown in Fig.V.5. The complex contains five types of subunit, and their apparent molecular weights, determined by comparison with standard proteins in the adjacent well, were: α-subunit, 54,000; β-subunit, 48,000; γ-subunit, 30,000; δ-subunit, 20,000; ε-subunit, 12,000. The apparent molecular weights obtained using the same standard proteins and a 7% acrylamide slab gel were generally higher; α-subunit, 56,000; β-subunit,51,000; γ-subunit, 34,500; δ-subunit, 21,500; ε-subunit, not determined. The same five subunits were present in the polypeptide profiles of the Mg-ATPases purified from the EACA- or low-ionic strength- washes, although some contaminating bands were usually present.

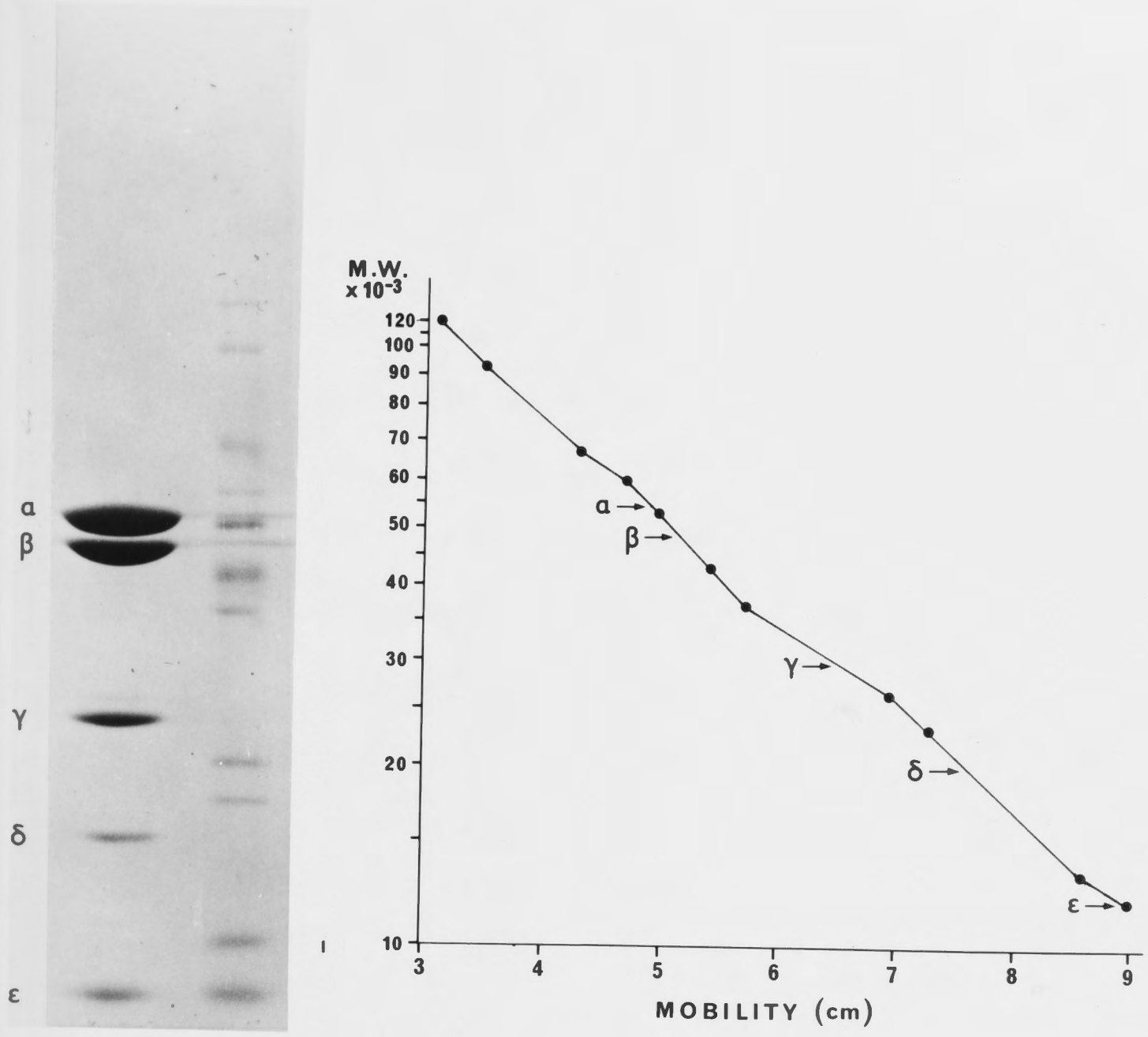


Figure V.5. SDS electrophoresis of the Mg-ATPase purified by two-dimensional electrophoresis from the Minus-PAB wash from strain AN248 (*unc*$^{+}$). The Mg-ATPase was purified from about 0.3mg of protein (1.3 units of Mg-ATPase activity). The gel piece containing Mg-ATPase activity was treated with SDS+mercaptoethanol at 100^{o}C, and then sealed into the well using agarose gel. The SDS gel was stained for protein. The positions of the Mg-ATPase subunits are indicated on the gel and on the accompanying graph of molecular weight against distance travelled in the gel. Molecular weight standard proteins (Stds) were: β-galactosidase (120,000), phosphorylase A (93,000), bovine serum albumin (67,000), catalase (60,000), glutamate dehydrogenase (53,000), ovalbumin (43,000), alcohol dehydrogenase (37,000), triosephosphate isomerase (26,500), adenylate kinase (23,000), ribonuclease A (13,500), cytochrome c (12,000).

(b) Determination of apparent isoelectric points of the subunits of the Mg-ATPase purified from the Minus-PAB wash

The major Mg-ATPase complex purified from the Minus-PAB wash was subjected to analytical isoelectric focusing as described in Chapter II.M(b). The excised gel containing Mg-ATPase was incubated at 100°C for 5 min in the SDS/mercaptoethanol buffer also used to solubilize membrane fractions. The treated gel piece was then sealed to an analytical isoelectric focusing gel using a gel of 1.5%(w/v) agarose in 0.67% Ampholine (pH 3.5 to 10) buffer containing 6M-urea. Solid urea was also added to raise the concentration to approximately 8-9M. Gels were then run in the normal way.

The apparent isoelectric points of the α-, β-, δ-, and ε-subunits of the 5-subunit Mg-ATPase from the Minus-PAB wash were estimated by slicing an isoelectric focusing gel into segments, measuring the pH gradient (Chapter II.M(b)), staining each segment, and reconstructing the gel. The composition of the Ampholines used in this experiment was 1:1:3 (pH ranges 3.5 to 10: 5 to 7: 4 to 6), and the apparent isoelectric points thus determined were α, pI 6.25; ε, pI 5.95; β, pI 5.35; and δ, pI 5.3 to 5.4. The identity of these subunits was confirmed by two-dimensional electrophoresis as described below. (see Fig.V.7).

Since the γ-subunit had an alkaline isoelectric point, gels covering the wide pH range were used to estimate its isoelectric point. Using gels containing the Ampholine composition 1:1:1:2 (pH ranges 3.5 to 5: 5 to 7: 7 to 9: 9 to 11) the pI of the γ-subunit was

estimated to be 8.9.

(c) Two-dimensional analytical electrophoresis of the Mg-ATPase purified from the Minus-PAB wash

The Mg-ATPase purified from the Minus-PAB wash was subjected to isoelectric focusing over the wide pH range, followed by second-dimension SDS electrophoresis. All five subunits are shown on the gel (Fig.V.6). The α-subunit is poorly focused, and in other gels, tended to streak out over the alkaline pH ranges without reaching pH 6.25, or occasionally focused at a higher apparent pI.

Similar preparations were subjected to electrophoresis over the acidic pH range (1:1:3; pH ranges 3.5 to 10: 5 to 7: 4 to 6) and the results are shown in Fig.V.7. The α-, β-, δ-, and ε-subunits are indicated. The gel shown in Fig.V.7a demonstrates the capacity and effectiveness of both the purification and the analytical methods. A total of 0.9mg of protein of the Minus-PAB wash was loaded onto two isoelectric focusing gels for purification. Both active regions from the slab gel were loaded onto a single analytical focusing gel, to give the two-dimensional pattern shown. A similar gel, containing much less protein, is shown in Fig.V.7b. The identity of the polypeptide on the acidic side of the δ-subunit (Fig.V.7a) was not established, although there is a polypeptide present in unwashed or PAB-washed membranes which runs in this position. The high molecular weight (∿160,000) species at an apparent pI of about 5.3 was invariably seen in two-dimensional gels, but not in one-dimensional SDS gels.

Occasionally, during the purification procedure the region

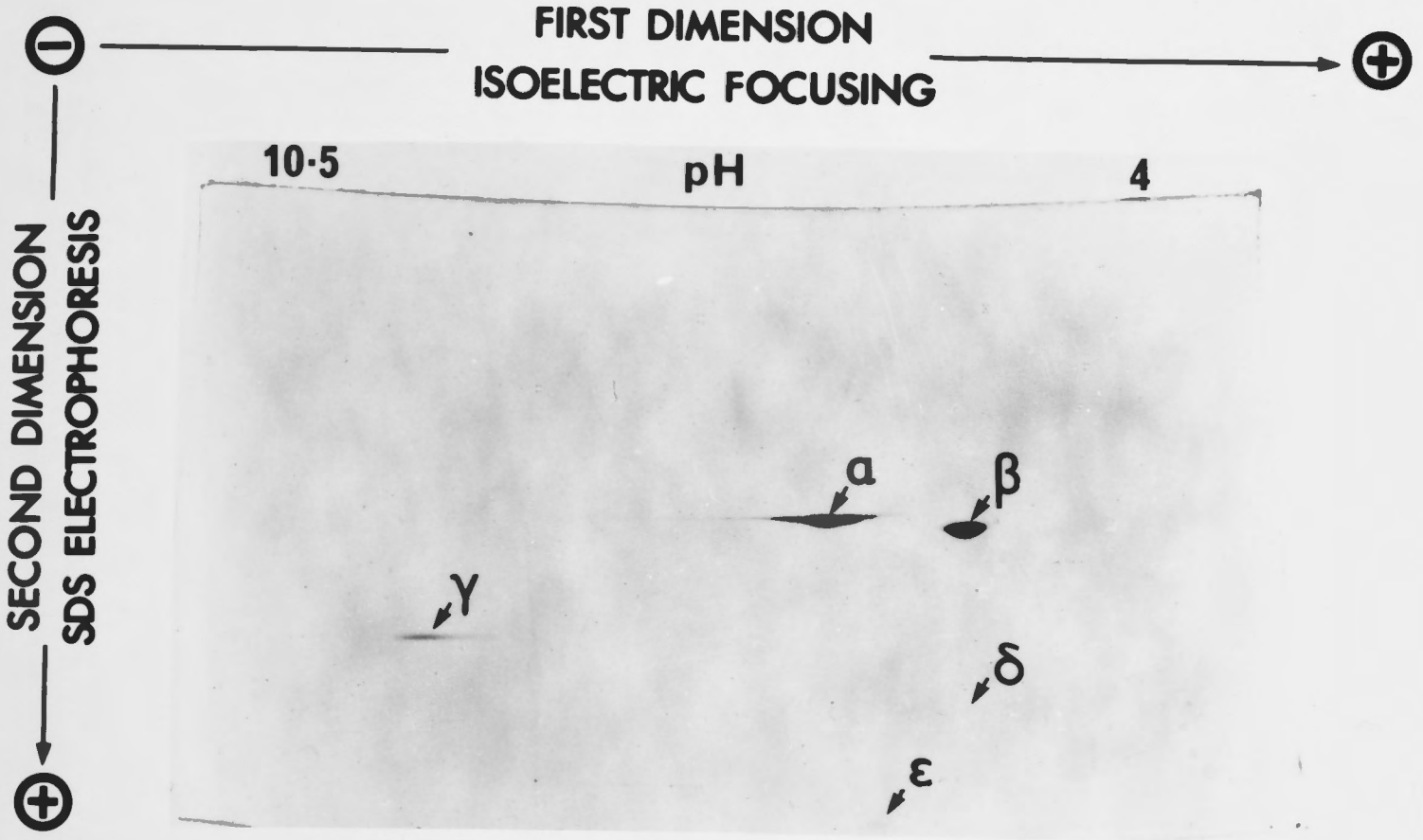


Figure V.6. Two-dimensional analytical gel electrophoresis of the Mg-ATPase purified from the Minus-PAB wash from strain AN248 (*unc*$^{+}$). Isoelectric focusing was performed over the wide pH range (iii) using Ampholines of the composition 1:1:1:2 (by volume) (pH ranges 3.5 to 5: 5 to 7: 7 to 9: 9 to 11). The pH range formed is shown. The cathode and anode solutions were 0.2M-NaOH and 10mM-H_3PO_4 respectively. Other details of the methods are given in Chapter II.L and M. The subunits of the Mg-ATPase are indicated. The sample used was the major species purified by gel electrophoresis from 1.3mg of protein of the Minus-PAB wash, containing 1.7 units of Mg-ATPase activity.

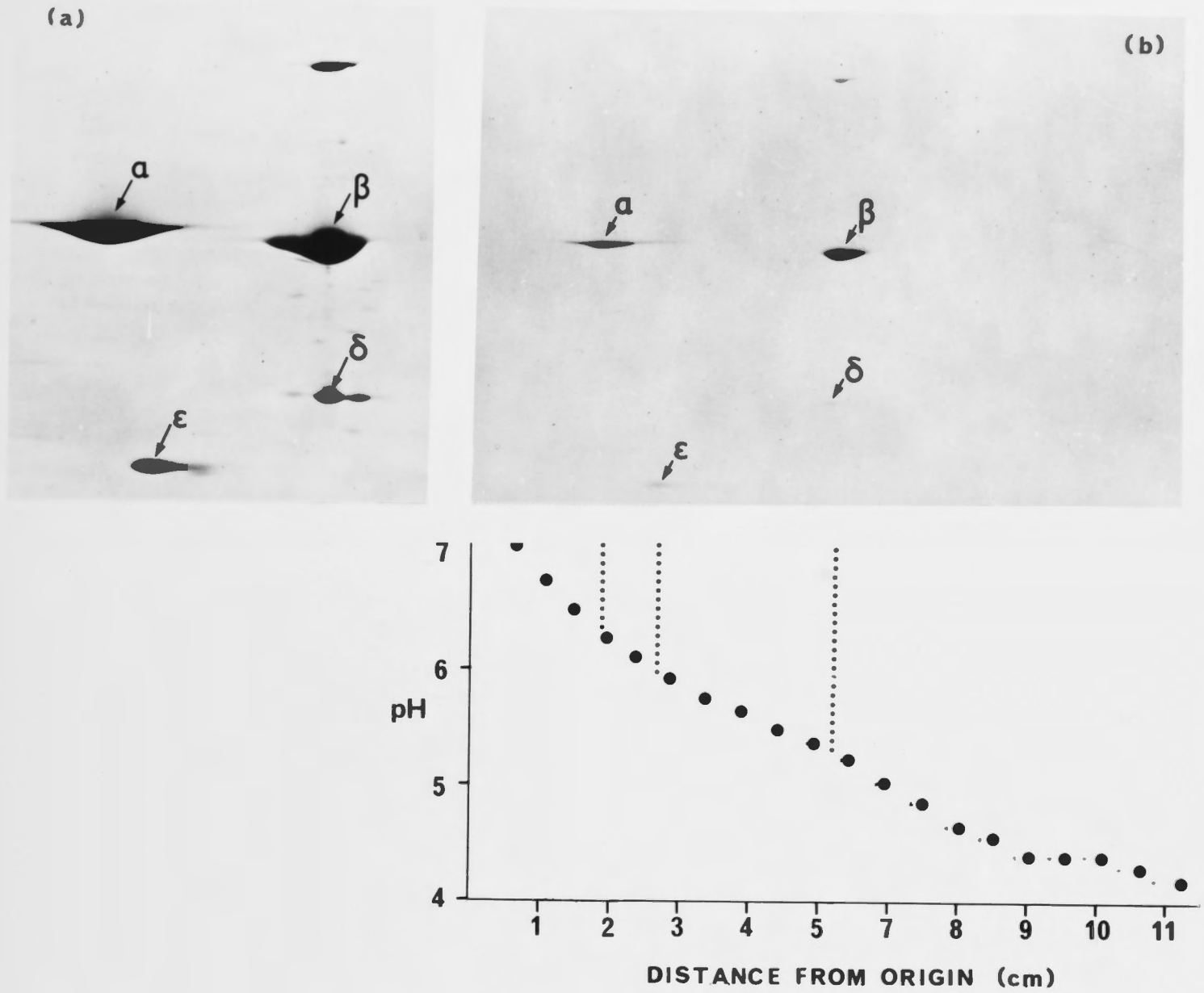


Figure V.7. Two-dimensional analytical gel electrophoresis of the Mg-ATPase complex purified from the Minus-PAB wash from strain AN248 (*unc*$^{+}$). The Ampholine composition (i) used in the first dimension was 1:1:3 (pH ranges 5 to 7: 3.5 to 10: 4 to 6). The Mg-ATPase was purified from the Minus-PAB wash, starting with: (a) 0.9mg of protein (5 units of Mg-ATPase activity); (b) 1.3mg of protein (1.7 units of Mg-ATPase activity). The latter preparation was made using shorter ultracentrifugation times than those described in the method and there was incomplete separation of membrane particles from the soluble fraction, hence the low specific activity and decreased efficiency of purification. The α-, β-, δ-, and ε-subunits are indicated. Details of the methods are given in Chapter II.L and M.

of gel containing Mg-ATPase activity appeared to divide incompletely in the second-dimension (see section C (c) (iii)). The upper and lower halves of such a spot were excised and run separately on two-dimensional analytical gels (Fig.V.8a and b). The complex of lower mobility during purification, contained a relatively strongly-stained δ-subunit band (Fig.V.8a) when compared with δ-subunit band from the higher-mobility complex (Fig.V.8b). This apparent difference was not investigated further.

E. ANALYSIS OF THE SUBUNITS OF THE Mg-ATPase COMPLEXES PURIFIED FROM THE CHLOROFORM-SOLUBILIZED PREPARATION FROM STRAIN AN248 (*unc*$^+$)

(a) Polypeptide composition of the purified chloroform-solubilized Mg-ATPase on single-dimensional SDS gels

Attempts were made to purify and analyse the chloroform-solubilized Mg-ATPase in the same way as described above for the low-ionic strength solubilized Mg-ATPase. However, although the purification procedure appeared to work satisfactorily, and the chloroform-solubilized complexes stained at rates similar to the complex from the Minus-PAB wash (cf., Fig.V.4), subsequent polypeptide analysis always revealed relatively small amounts of the subunits of the purified chloroform-solubilized Mg-ATPase.

For this reason, it was necessary to severely overload the purification system to obtain enough Mg-ATPase to determine the

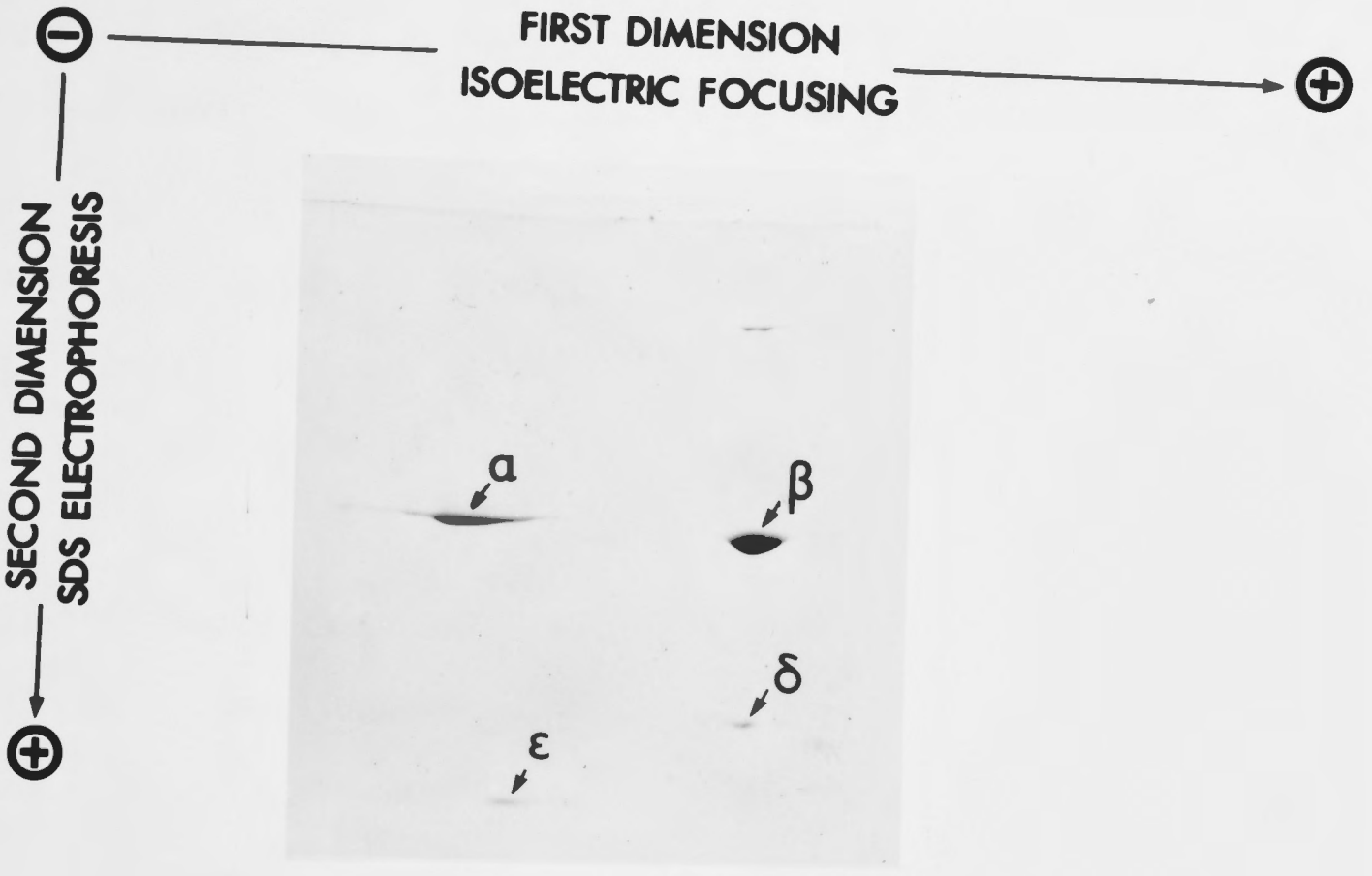


Figure V.8. Two-dimensional analytical gel electrophoresis of the Mg-ATPase complex purified from the Minus-PAB wash from strain AN248 (*unc*$^{+}$). The Ampholine composition (i) used in the first dimension was 1:1:3 (pH ranges 5 to 7: 3.5 to 10: 4 to 6). The Mg-ATPase was purified from the Minus-PAB wash, starting with 0.6mg of protein (3.3 units of Mg-ATPase activity). The top (a) and bottom (b) halves of the active region were excised and subjected to analytical two-dimensional electrophoresis. The α-, β-, δ- and ε-subunits are indicated. Details of the methods are given in Chapter II.L and M.

polypeptide composition. The high and low molecular weight complexes were consequently poorly resolved. In an attempt to determine the subunit structure of these complexes, the areas of the gel containing the high molecular weight ('340,000') complex (Fig.V.9b), the low molecular weight ('310,000') complex (Fig.V.9c), or both complexes (Fig.V.9a) were excised, treated with SDS + mercaptoethanol, and subjected to single-dimensional SDS electrophoresis. Because of the impurity of these preparations, it was not possible to establish their complete subunit structure, except by two-dimensional electrophoresis as described later in this chapter. Nevertheless, the α-, β-, and γ-subunits are clearly present (Fig.V.9). The high molecular weight complex (Fig.V.9b) appeared to contain greater amounts of a polypeptide of 12,000 daltons than the low molecular weight complex (Fig.V.9c), as judged by the staining intensities relative to those of the major subunits. This polypeptide may be the ε-subunit.

(b) Re-examination of the protein composition of the crude chloroform-solubilized Mg-ATPase preparation

The finding that only small amounts of the Mg-ATPase subunits were obtained from the purified chloroform-solubilized Mg-ATPase seemed to be incompatible with the evidence that the major proteins present in the crude preparation were the two types of Mg-ATPase complex. Firstly, the preparation had a high specific Mg-ATPase activity. Secondly, the purified Mg-ATPase stained readily in the ATP/lead stain. Thirdly, the major polypeptides present in the SDS-treated preparation were the α- and β-subunits of the Mg-ATPase.

However, a re-examination of non-dissociating gels which

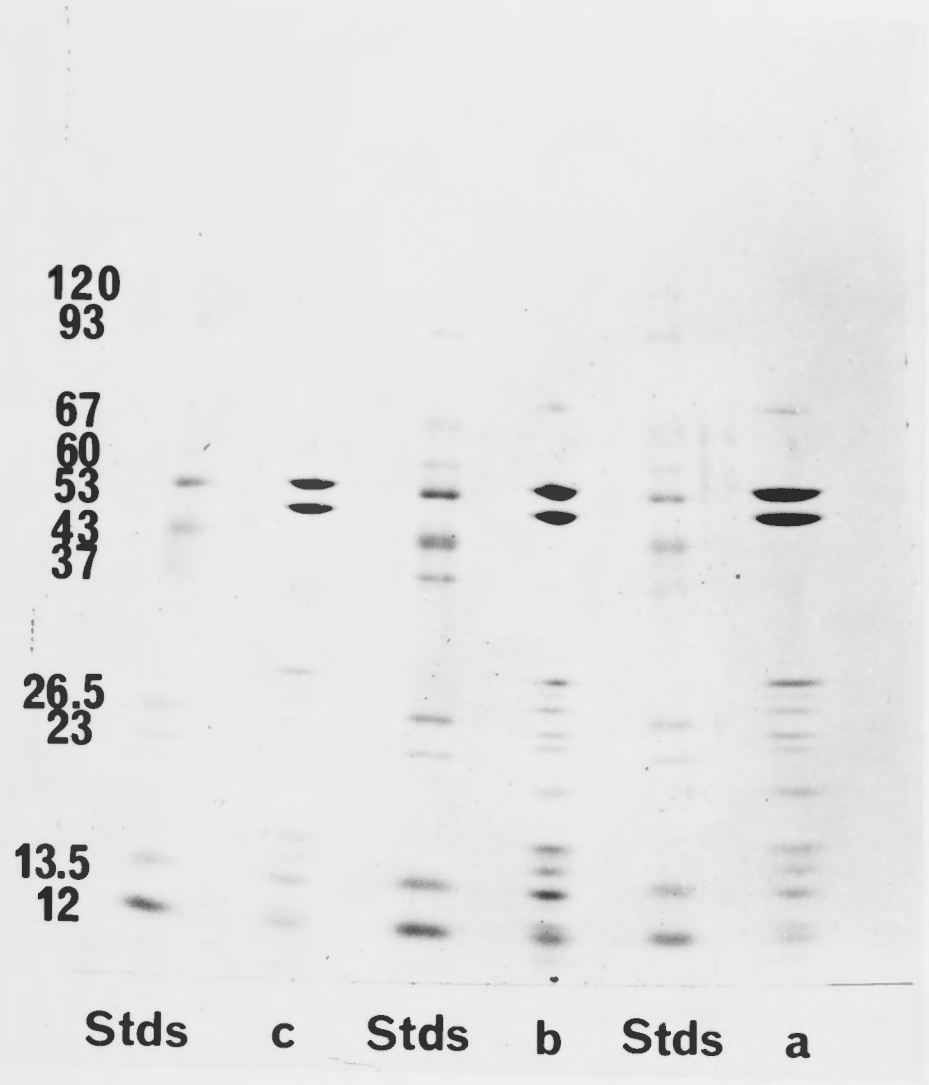


Figure V.9. SDS electrophoresis of the Mg-ATPase purified by two-dimensional electrophoresis from the chloroform-solubilized preparation from strain AN248 (*unc*$^{+}$). Each well contained Mg-ATPase purified from about 4.5mg of protein of the crude preparation. The whole active area (a), or the top half (b), or the bottom half of the area (c) was excised, treated with SDS + mercaptoethanol at 100^{o}C, and then sealed into the well using agarose gel. The SDS gel was stained for protein. The molecular weight standard proteins (Stds) were: β-galactosidase (120,000), phosphorylase A (93,000), bovine serum albumin (67,000), catalase (60,000), glutamate dehydrogenase (53,000), ovalbumin (43,000), alcohol dehydrogenase (37,000), triosephosphate isomerase (26,500), adenylate kinase (23,000), ribonuclease A (13,500), cytochrome c (12,000). Molecular weights (x10^{-3}) of the protein standards are indicated.

had been stained for protein (e.g. Fig.V.10) revealed the presence of three bands of high mobility. When the data from several gels were collated, a standard curve could be drawn, and the apparent molecular weights of these proteins estimated using this curve, suggested that they were the individual α-, β- and γ-subunits of the Mg-ATPase (see Fig. V.3).

(c) Two-dimensional analytical electrophoresis of purified, chloroform-solubilized Mg-ATPase

Attempts were made to show by two-dimensional analytical electrophoresis the presence or absence of the δ- and ε-subunits in the partially-purified preparation shown in Fig.V.9a. The Mg-ATPases were purified from a total of 6.7mg of protein, and the gel pieces were loaded onto a single wide pH range analytical isoelectric focusing gel. The two-dimensional pattern obtained is shown in Fig.V.11. The α-, β- and γ-subunits were present in the normal positions, and the ε-subunit was also present (Fig.V.11). However, no δ-subunit was detectable. The band at a molecular weight of 20,000 on the one-dimensional SDS gels (Fig.V.9) was a protein with a very alkaline isoelectric point (Fig.V.11).

F. DISCUSSION

Evidence has been presented from gel electrophoresis experiments that the Mg-ATPase complexes solubilized from normal membranes by chloroform treatment and by low-ionic strength washing, are different. Firstly, the chloroform-solubilized preparation contains two major Mg-ATPase species separable on gels, whereas the

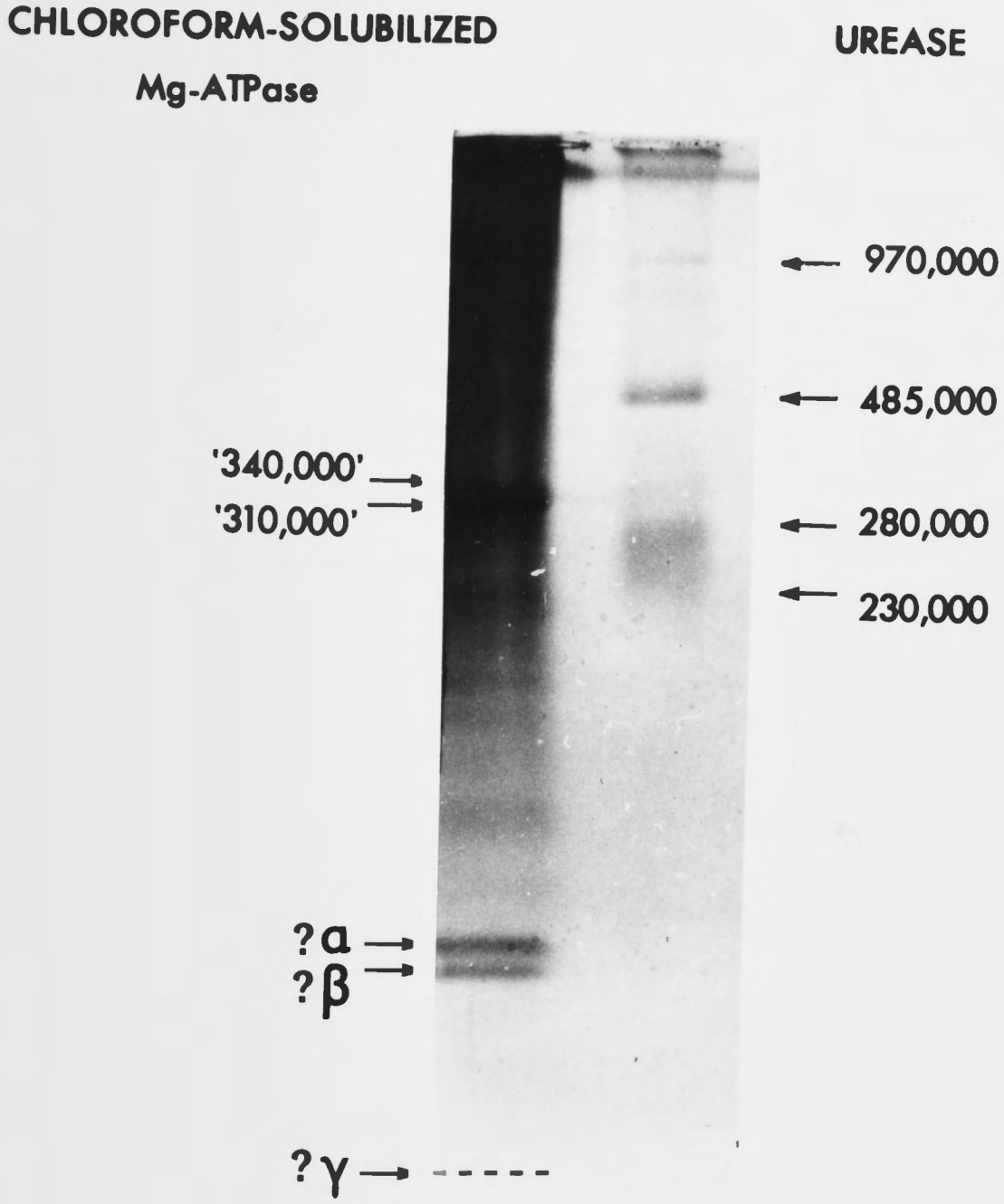


Figure V.10. Comparison of the mobilities of the chloroform-solubilized Mg-ATPases from strain AN248 (*unc*$^{+}$) with the mobility of jack bean urease and its dimer, showing the possible breakdown of the chloroform-solubilized Mg-ATPase into subunits. The various bands are indicated. A sharp band, labelled '?γ', was present at the position indicated, which was in the portion of the gel which fragmented during electrophoresis (see Fig. V.4). The gel (T=5.7-8.6%, C=2.6%) was run at 20mA for 12 hours. The data from this gel were also included in Fig. V.3. The fuzzy band in the urease pattern between approximately 280,000 and 230,000 daltons (see Fig. V.3) is probably the 240,000 dalton form of urease (see Contaxis and Reithel, 1971).

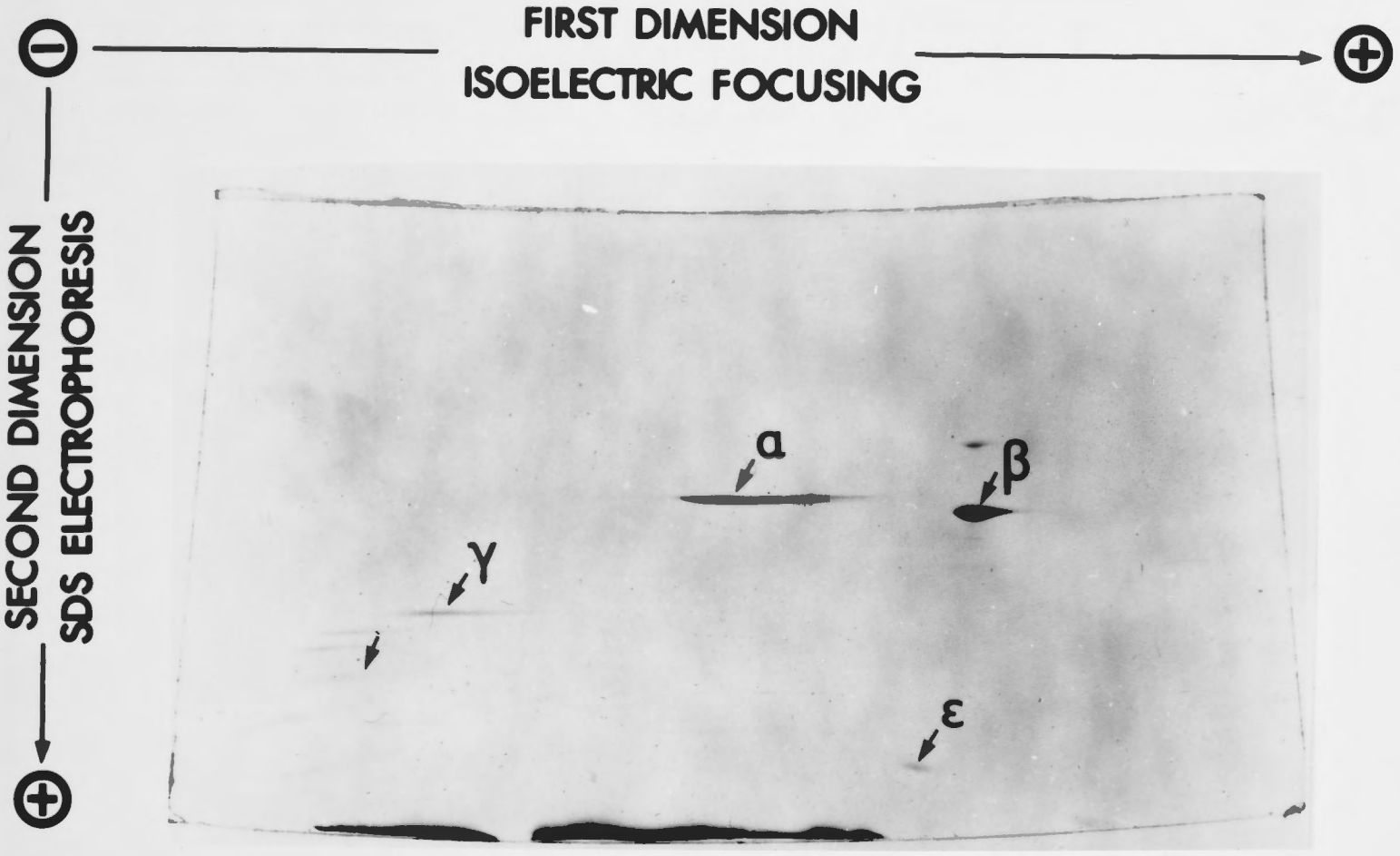


Figure V.11. Two-dimensional analytical gel electrophoresis of the purified chloroform-solubilized Mg-ATPase from strain AN248 (*unc*$^+$). The Ampholine composition (iii) used in the first dimension was 1:1:1:2 (pH ranges 3.5 to 5: 5 to 7: 7 to 9: 9 to 11). Complete areas containing active Mg-ATPase were excised from two two-dimensional gels, starting with a total of 6.7mg of protein of the chloroform-solubilized preparation. The gel pieces were treated with SDS+mercaptoethanol at 100°C before analytical electrophoresis. The α-, β-, γ-, and ε-subunits are indicated, as well as the 20,000 dalton component discussed in the text. Details of the methods are given in Chapters L and M.

Mg-ATPase solubilized by low-ionic strength treatment can be separated into at least one major component, as well as a minor one. Secondly, the major species of Mg-ATPase solubilized by low-ionic strength treatment focuses over a wider and slightly different pH range, and has a lower mobility in the second dimension, than either of the chloroform-solubilized Mg-ATPases. Thirdly, the δ-subunit, which is present in the Mg-ATPase solubilized by low-ionic strength washing, appears to be missing from the chloroform-solubilized enzymes.

The finding that the chloroform-solubilized Mg-ATPase could not reconstitute NADH- or ATP-driven atebrin fluorescence quenching in membranes stripped of Mg-ATPase, whereas the low-ionic strength solubilized Mg-ATPase was able to restore these activities, can thus be explained on a structural basis. All five types of subunit must be present in the complex before it will bind to stripped membranes to restore NADH- and ATP-induced atebrin fluorescence quenching. Other workers have also found that all five types of subunit are necessary for the reconstitution of energy-linked activities. Preparations of the Mg-ATPase containing no δ-subunit have been shown to be unable to reconstitute, in stripped membranes, either the ATP-driven transhydrogenase reaction (Bragg *et al*, 1973; Kobayashi and Anraku, 1974; Futai *et al*, 1974; Bragg and Hou, 1975) or oxidative phosphorylation (Smith and Sternweis, 1975; Sternweis and Smith, 1977), or the quenching of acridine dye fluorescence (Nelson *et al*, 1974). Such preparations were unable to bind to the membranes, as assessed by lack of sedimentation of the Mg-ATPase activity during centrifugation, or by the lack of reconstitution of

respiration-driven transhydrogenase in stripped membranes (Bragg and Hou, 1972; Bragg *et al*, 1973; Futai *et al*, 1974; Smith and Sternweis, 1975, 1977). Bragg *et al* (1973) and Futai *et al* (1974) prepared Mg-ATPase containing all five types of subunit, and found that it was able to restore both the ATP-driven and the respiration-driven transhydrogenase activities in stripped membranes, but that the Mg-ATPase lacking the δ-subunit could not. Preparations of Mg-ATPase containing all five types of subunit have been extensively used to reconstitute energy-linked activities (Bragg and Hou, 1972, 1975; Tsuchiya and Rosen, 1975; Smith and Sternweis, 1975, 1977; Smith *et al*, 1975; Vogel and Steinhart, 1976; Sternweis and Smith, 1977).

The conclusion has often been drawn that the δ-subunit is the structural connection between the (α+ β + γ +ε) complex and the membrane sector (Smith and Sternweis, 1975, 1977; Smith *et al*, 1975; Sternweis and Smith, 1977; Vogel and Steinhart, 1976). However, the evidence for this interpretation is not strong. The observation is that the Mg-ATPase is unable to bind to the membrane unless it contains all five types of subunit. There is thus no necessity to invoke a special role for the δ-subunit. However, Yoshida *et al* (1977), using the TF_1-ATPase and TF_o (membrane sector) preparations from the thermophilic bacterium PS3, have presented evidence that the δ- and ε-subunits can bind independently to vesicles containing TF_o, but that both the δ- and ε-subunits must be bound to the membrane before the γ-subunit can bind. A complex containing only the (γ+δ+ε) subunits was sufficient to block the proton channel in these vesicles (Yoshida *et al*, 1977), indicating that the organization of these subunits after rebinding was the same as that in the complete Mg-ATPase complex. Sternweis and Smith (1977) claimed that, in *E.coli*, the δ-subunit does

not by itself bind to the membranes, but they presented no evidence for this opinion.

The Mg-ATPase complex solubilized by the Minus-PAB washing procedure is a protein of about 360,000 daltons, and it consists of five types of subunit, with the apparent molecular weights: α, 54,000; β, 48,000; γ, 30,000; δ, 20,000; and ε, 12,000. The apparent molecular weights were up to 4,500 daltons higher when the subunits were separated on a 7% acrylamide SDS gel instead of the pore-gradient SDS gel, against the same standard proteins. In the 7% acrylamide gel system, the ability of the polypeptides to bind amounts of SDS proportional to their molecular weights would largely determine the apparent molecular weights obtained. In pore-gradient gels, molecular seiving of the denatured polypeptide chains probably has a greater influence on the separation, and therefore molecular weight values obtained using this system are considered more reliable. The molecular weights obtained by other workers for the Mg-ATPase complex and for its subunits are listed in Tables V.1 and 2. These values are generally similar to those obtained in the present work, when the limitations of the various methods are taken into account. Futai *et al* (1974) noted a difference in molecular weight between the Mg-ATPases which could and those which could not reconstitute energy-linked activities (Table V.1).

The apparent isoelectric points of the intact Mg-ATPase complexes - pI 5.1 to 5.3 for the chloroform-solubilized Mg-ATPases and

TABLE V.1 The molecular weights determined for the Mg-ATPase complex from *Escherichia coli*

MOLECULAR WEIGHTS ($\times 10^{-3}$)	METHOD OF DETERMINATION	REFERENCE
WITH δ-SUBUNIT		
360	Pore-gradient electrophoresis	Present work
296	Equilibrium gradient centrifugation	Futai *et al* (1974)
350	Gel filtration	Giordano *et al* (1975)
370	Extrapolated from δ-subunit molecular weight	Sternweis & Smith (1977)
? δ-SUBUNIT		
365 390	Gel filtration (2 estimates)	Davies & Bragg (1972)
300	Gel filtration	Carriera *et al* (1973)
400 (1)	Gel electrophoresis	Abrams & Smith (1974)
350	Gel filtration	Adler & Rosen (1976)
340	Pore-gradient electrophoresis	Vogel & Steinhart (1976)
WITHOUT δ-SUBUNIT		
340 310	Pore-gradient electrophoresis	Present work
400-600	Gel filtration	Kobayashi & Anraku (1972)
360	Sedimentation velocity	Hanson & Kennedy (1973)
240	Equilibrium gradient centrifugation	Futai *et al* (1974)

(1) A lower molecular weight active form was obtained during gel filtration.

TABLE V.2 The molecular weights determined for the subunits of the Mg-ATPase from *E.coli*

MOLECULAR WEIGHTS ($\times 10^{-3}$)						
α	β	γ	δ	ε	METHOD OF DETERMINATION (1)	REFERENCE
54	48	30	20	12	Pore-gradient SDS (T 7.5-22.5%)	Present work
56	51	34.5	21.5	n.d.	SDS (T 7%)	Present work
56.8	51.8	30.5	21	11.5	SDS (T 7.5%)	Bragg & Hou (1972)
56.8	51.8	32	20.7	13.2	SDS (T 7.5%)	Bragg & Hou (1975)
60	56	35	abs	13	SDS (T 10%)	Hanson & Kennedy (1973)
54	52	33	abs	11	SDS (T 10%)	Kobayashi & Anraku (1974)
58	52	31	20	12	SDS (T 4.5%)	Futai *et al* (1974)
56	52	32	21	11.5	SDS (T 7.5%)	Vogel & Steinhart (1976)
54	48	30	21	13	SDS (T 7%)	Azocar & Muñoz (1977)
66	60	28	abs	13	SDS (T 7%)	Azocar & Muñoz (1977)
			35(2)	16	Gel filtration	Smith & Sternweis (1977)
			18.5	16	SDS (T 12.9%) SDS/urea (T 13.3%) Sedimentation equilibrium	Sternweis & Smith (1977)
				9.5 (19) (39)	SDS (T 12%)	Nieuwenhuis & Bakkenist (1977)

Abbreviations: abs = absent, n.d. = not determined.

(1) SDS gel electrophoresis is denoted 'SDS'. The (acrylamide+bisacrylamide) concentration of the gel is given as T%. Gels were calibrated using a variety of standard proteins.

(2) Value amended to 18500 by Sternweis and Smith (1977). Higher apparent molecular weight though to be due to elongated shape of δ-subunit.

pI 5.1 to 5.5 for the low-ionic strength-solubilized complexes - are higher than the isoelectric point of 4.6 reported by Kobayashi and Anraku (1974). However, the sample was placed at the anode in the experiment of Kobayashi and Anraku (1974), and at the cathode in the present work, and in both cases, apparent precipitation of the enzyme occurred. The true isoelectric points may therefore lie somewhere between the two values. The Mg-ATPase purified by Kobayashi and Anraku (1974) contained no detectable δ-subunit. The apparent isoelectric points of the subunits of the Mg-ATPase, also determined by isoelectric focusing, were: α, pI 6.25; β, pI 5.35; γ, pI 8.9; δ, pI 5.3 - 5.4; and ε, pI 5.95.

In two-dimensional analytical gels, a high molecular weight species was present at a molecular weight of about 160,000 and a pI of 5.3. This species was not seen in single-dimensional SDS gels. In the isoelectric focusing gels, the region around pH 5.3 would contain high concentrations of the β- and δ-subunits, and the high molecular weight spot may possibly be an aggregate containing one or both of these subunits. Aggregates ('dimers' and 'trimers') were formed in small amounts by bovine serum albumin monomers in the presence of SDS, and aggregates of unidentified polypeptides were also noticed after second-dimensional SDS electrophoresis with acetic acid-urea gels in the first dimension (Chapter III.C). Nieuwenhuis and Bakkenist (1977) showed that the ε-subunit of the Mg-ATPase forms aggregates in the presence of SDS. Recently, Berden and Voorn-Brouwer (1978) reported the dimerization of the DCCD-binding protein from beef heart mitochondria in SDS, but not in SDS + urea, gels.

The major species of Mg-ATPase present in the concentrated

Minus-PAB wash may actually be two types of complex, differing slightly in their mobilities in slab gels, and possibly differing in their content of δ-subunit. The loss of a δ-subunit (apparent pI 5.3 - 5.4) from the complex (apparent pI 5.1 - 5.5) would not be expected to alter the isoelectric point, in agreement with the observation that there is no detectable separation of two such species in the first dimension. Several workers have found that the δ-subunit is easily lost during purification of the Mg-ATPase from some strains of *E.coli* (Bragg *et al*, 1973; Futai *et al*, 1974; Vogel and Steinhart, 1976; Sternweis and Smith, 1977), and it may be that one or more δ-subunits can be dissociated from the rest of the complex during the electrophoresis procedure (see also Azocar and Munoz, 1977), despite the presence of Mg^{2+} in the gel. Mg^{2+} ions had been included in the gel system after Abrams *et al* (1976) reported that the Mg^{2+} was essential to bind the δ-subunit to the rest of the ATPase from *Streptococcus faecalis*. However, Sternweis and Smith (1977) have shown that Mg^{2+} is not necessary for the binding of the δ-subunit to the (α+β+γ+ε) Mg-ATPase of *E.coli*. These workers have also concluded that there is only one δ-subunit bound per complex of 370,000 daltons (Sternweis and Smith, 1977).

As observed above, the lack of a δ-subunit would not be expected to affect the isoelectric focusing of the Mg-ATPase. However, the chloroform-solubilized Mg-ATPase species which lack the δ-subunit, focus over a more acidic mean pH range than the complex containing five types of subunit. Moreover, the '310,000 dalton' complex focuses at a more acidic apparent pI than the '340,000 dalton' Mg-ATPase, also solubilized by chloroform treatment. One explanation for these results might be that the '340,000' complex lacks one ε-subunit (pI 5.95),

whilst the '310,000' complex lacks two ε-subunits. The loss of these subunits would be consistent with the observed effect on the isoelectric focusing of the resulting Mg-ATPase complexes.

Attempts to test such an hypothesis were hampered by the apparent dissociation of the Mg-ATPases released by chloroform treatment, into their constituent subunits. It was not clear from the present work whether the dissociation occurred during the chloroform extraction procedure and storage of the preparation, or during gel electrophoresis. The recovery of Mg-ATPase activity after chloroform treatment (33%) was lower than that obtained using the EACA-washing procedure (57%) despite the higher specific activity of the former preparation (Cox *et al*, 1978b). Moreover it is possible that the high specific Mg-ATPase activity of the chloroform-solubilized preparation was in part due to activation of the enzyme. Certainly, after purification by gel electrophoresis, the intact complexes react readily with ATP in the presence of Mg^{2+} (and Pb^{2+}) during the staining procedure, at rates similar to those obtained with low-ionic strength-solubilized Mg-ATPase containing far greater amounts of protein.

The ε-subunit has been claimed to be an "ATPase inhibitor", since preparations containing the isolated ε-subunit inhibit the Mg-ATPase activity of solubilized Mg-ATPase (Smith *et al*, 1975; Smith and Sternweis, 1977; Nieuwenhuis and Bakkenist, 1977). However, the Mg-ATPase complexes used in these experiments already contained the ε-subunit before the addition of more ε-subunit, and so the significance of these results is unclear. Evidence is lacking that the ε-subunit is moved from its 'inhibitory' site to a 'non-inhibitory' site during solubilization of the Mg-ATPase from the membrane, as has been suggested by Smith and Sternweis (1977), Smith *et al* (1975), and Nieuwenhuis and

Bakkenist (1977). A similar idea was proposed by Nelson *et al* (1972) and Van de Stadt *et al* (1973) for the chloroplast and beef heart Mg-ATPases respectively. Trypsin is known to stimulate Mg- or Ca-ATPase activity, and to digest the δ-, ε- and most of the γ-subunits (Bragg and Hou, 1975). It remains to be established, however, whether or not activation of the enzyme occurs following the removal of the ε-subunit(s) from the enzyme, as may have occurred in the present work.

Risi *et al* (1977) found two species of Mg-ATPase from a strain of the bacterium *Micrococcus*, separable by gel electrophoresis. The low mobility complex contains α-, β-, γ- and δ-subunits, whilst the higher mobility complex lacks the δ-subunit. Adolfsen *et al* (1975), working with the Mg-ATPase from the bacterium *Alcaligenes faecalis*, have reported five species of active Mg-ATPase, separable by gel electrophoresis. The *A.faecalis* type "IA" and "IB" Mg-ATPases, which bind to stripped membranes, appear to lack the δ-subunit. Type "IIA", "IIB" and "III" Mg-ATPases are unable to bind to membranes, and also lack the δ-subunit, but the ε-subunit is still present in each of these types. The order of mobility, starting with the slowest, was IA, IB, IIA, IIB, and III. The relationship between these results and those of the present work is not clear. Adolfsen *et al* (1975) also found that the chloroplast CF_1-ATPase consisted of two species separable on gels, and this also appeared to be the case for beef heart F_1-ATPase.

A protein which was thought to be the ε-subunit of the Mg-ATPase of *A. faecalis* was found to have an inhibitory effect on Mg-ATPase activity (Adolfsen *et al*, 1975). The protein was thought to be very basic, and Adolfsen and Moudrianakis (1976) have since shown that poly-cationic molecules are potent inhibitors of the Mg-ATPase.

No attempt was made by these workers to ascertain whether such molecules simply sequester ATP^{4-}. In any case, the present work suggests that the ε-subunit of the *E.coli* Mg-ATPase (pI 5.95) is unlikely to act in this way.

Chapter VI

CHARACTERIZATION OF THE MUTANT *uncD* GENE PRODUCT IN STRAINS CARRYING THE *uncD409* ALLELE

A. THE *uncD409* ALLELE: INTRODUCTION

The isolation of a strain carrying the *uncD409* allele was described by Cox *et al* (1978a). A mutant strain unable to grow on the non-fermentable carbon source, succinate, was isolated after mutagenesis using N-methyl-N'-nitro-N-nitrosoguanidine. A mutant allele (*unc-409*) was transferred by co-transduction with the *ilv* locus into strain AN248 (*ilvC*), to form the new strain AN463 (ilv^+, *unc-409*). This strain was found to have a low aerobic growth yield, characteristic of uncoupled strains, when grown in limiting concentrations of glucose (F. Gibson, unpublished work). It was shown by Cox *et al* (1978a) that the *unc-409* allele, introduced on a plasmid into recombination-deficient strains carrying either the *uncA401*, *uncB402* or *uncC424* allele, could complement each of these mutations. The *unc-409* allele therefore affected a gene distinct from the *uncA*, *uncB* or *uncC* genes, and this gene was designated *uncD* (Cox *et al*, 1978a).

Membranes from strains carrying the *uncD409* allele had lowered P/O ratios, no detectable Mg-ATPase activity, and no ATP-dependent transhydrogenase activity or ATP-dependent quenching of atebrin fluorescence (Cox *et al*, 1978a). NADH-induced atebrin fluorescence quenching was normal (Cox *et al*, 1978a).

Work to be described below leading to the identification of

the defective polypeptide in strain AN463 (*uncD409*) was done concurrently with further characterization of the strain carried out by J. A. Downie, G. B. Cox, and J. Radik. Details of their work will be given where appropriate.

B. DETECTION OF THE Mg-ATPase SUBUNITS IN TWO-DIMENSIONAL GELS OF THE POLYPEPTIDES OF NORMAL MEMBRANES

Using the purified Mg-ATPase obtained as described in Chapter IV, together with the analytical electrophoresis techniques outlined in Chapter III, it was possible to identify the major subunits of the Mg-ATPase in complex polypeptide patterns on two-dimensional gels. Membrane preparations were treated with SDS and mercaptoethanol at 100°C, and subjected to two-dimensional electrophoresis. The positions of the α- and β-subunits were located by co-electrophoresis of PAB-washed membranes and a purified Mg-ATPase from strain AN248 (*unc*$^+$), and by comparison of the resulting gels with a gel of the membranes alone (Fig.VI.1). The polypeptide corresponding to the δ-subunit could not be identified unequivocally. The region in which the ε-subunit would be found was badly affected by streaking of polypeptides (cf.Fig.III.4a). The membrane polypeptides from *unc* mutant strains could then be analysed, and this chapter describes such an analysis of strains carrying the *uncD409* allele.

C. POLYPEPTIDE PATTERNS OF WASHED MEMBRANES FROM STRAINS AN463 (*uncD409*) AND AN248 (*unc*$^+$)

The polypeptide composition of the PAB-washed membranes from

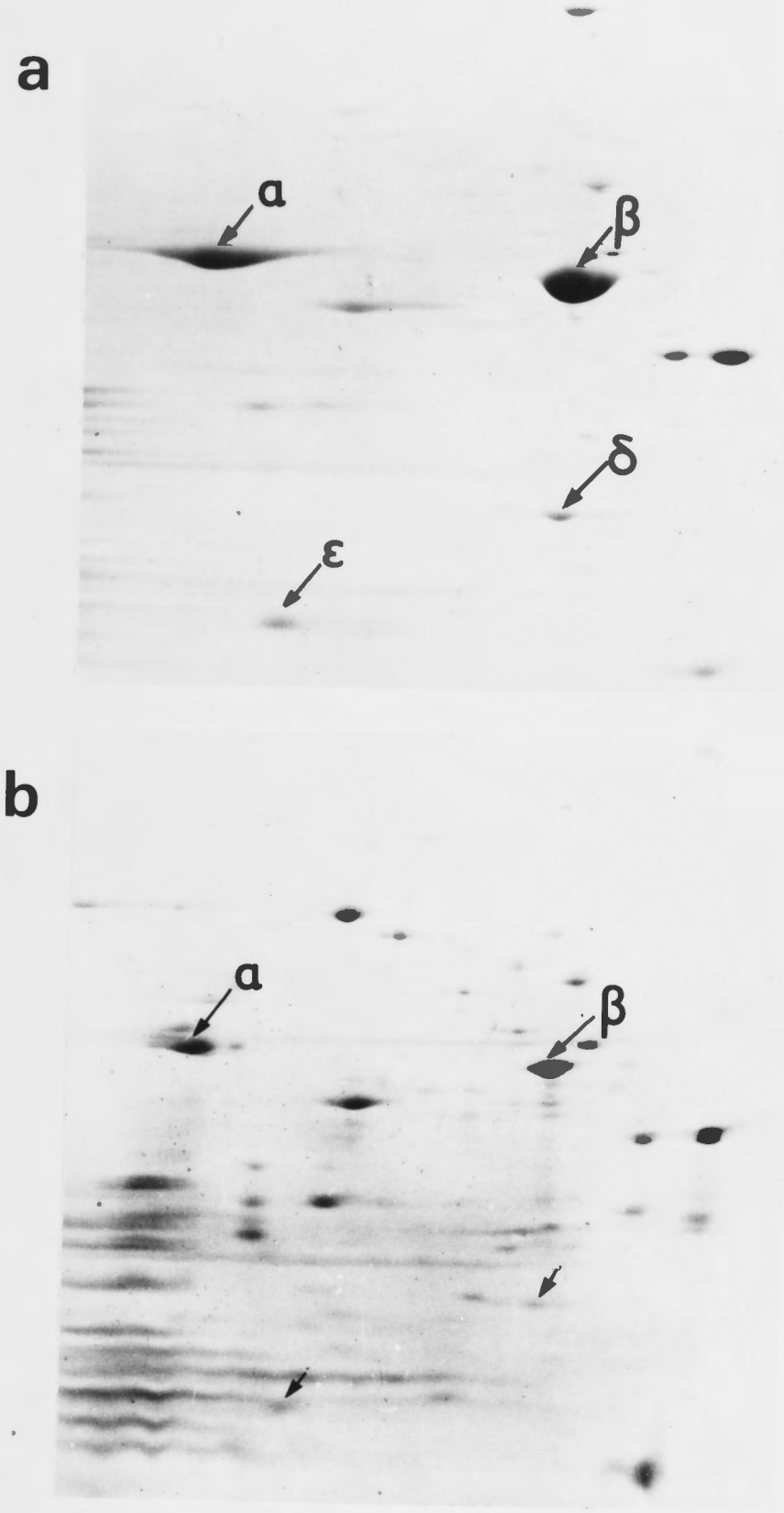


Figure VI.1 Two-dimensional analytical gel electrophoresis of PAB-washed membranes (b) and PAB-wash membranes plus 5-subunit Mg-ATPase (a) from strain AN248 (*unc*$^{+}$). Sample (a) contained 196μg of protein of the PAB-washed membrane fraction, plus purified Mg-ATPase in a small piece of SDS-treated semi-preparative gel. Sample (b) contained 196μg of protein of the PAB-washed membranes only. The Ampholine composition used in the first dimension gels was 1:1:3 (pH ranges 3.5 to 10: 5 to 7: 4 to 6). The positions of the α- β-, δ- and ε-subunits are indicated, as well as the polypeptides tentatively identified as the δ- and ε-subunits. The pH gradient and molecular weight scales are shown. Details of the methods can be found in Chapter II.

strain AN248 (*unc*$^+$) includes the α- and β-subunits of the Mg-ATPase (Fig.VI.2a). The EACA-washed membranes have lost most of their Mg-ATPase activity, and correspondingly, only small amounts of the α- and β-subunits of the Mg-ATPase are observed in the gels (Fig.VI.2b).

Membranes from strain AN463 (*uncD409*) were fractionated in the absence (Scheme IV.1) or in the presence (Scheme IV.2b) of PAB, even though these membranes lacked Mg-ATPase activity. The fractionation was carried out by G. B. Cox, J. Radik, B. Webb and B. Homer. The polypeptides of PAB-washed membranes of strain AN463 (*uncD409*) do not include the normal β- and α-subunits (Fig.VI.2c). There is, however, a polypeptide with the same apparent molecular weight as the β-subunit (48,000), but with an apparent isoelectric point about 0.1pH units higher than that of the normal β-subunit which focused at pI 5.35 (see Fig.V.7). The difference between the isoelectric points was confirmed by electrophoresis of a mixture of PAB-washed membranes from strains AN463 (*uncD409*) and AN248 (*unc*$^+$) (Fig.VI.2e). Furthermore, the abnormal β-subunit was also present in the EACA-washed membranes from strain AN463 (*uncD409*) (Fig.VI.2d) in apparently undiminished amounts. The α-subunit was tentatively identified in the cytoplasmic fraction (results not shown).

D. DETECTION OF THE NORMAL AND ABNORMAL β-SUBUNITS OF THE Mg-ATPase IN THE PARTIAL DIPLOID STRAIN AN821 (*uncD409/unc*$^+$)

It was thus apparent that strain AN463 (*uncD409*) did not form an aggregate corresponding to an inactive Mg-ATPase complex. It was of interest to ascertain whether the abnormal β-subunit from the *uncD409* strain could assemble into such an aggregate in the presence of

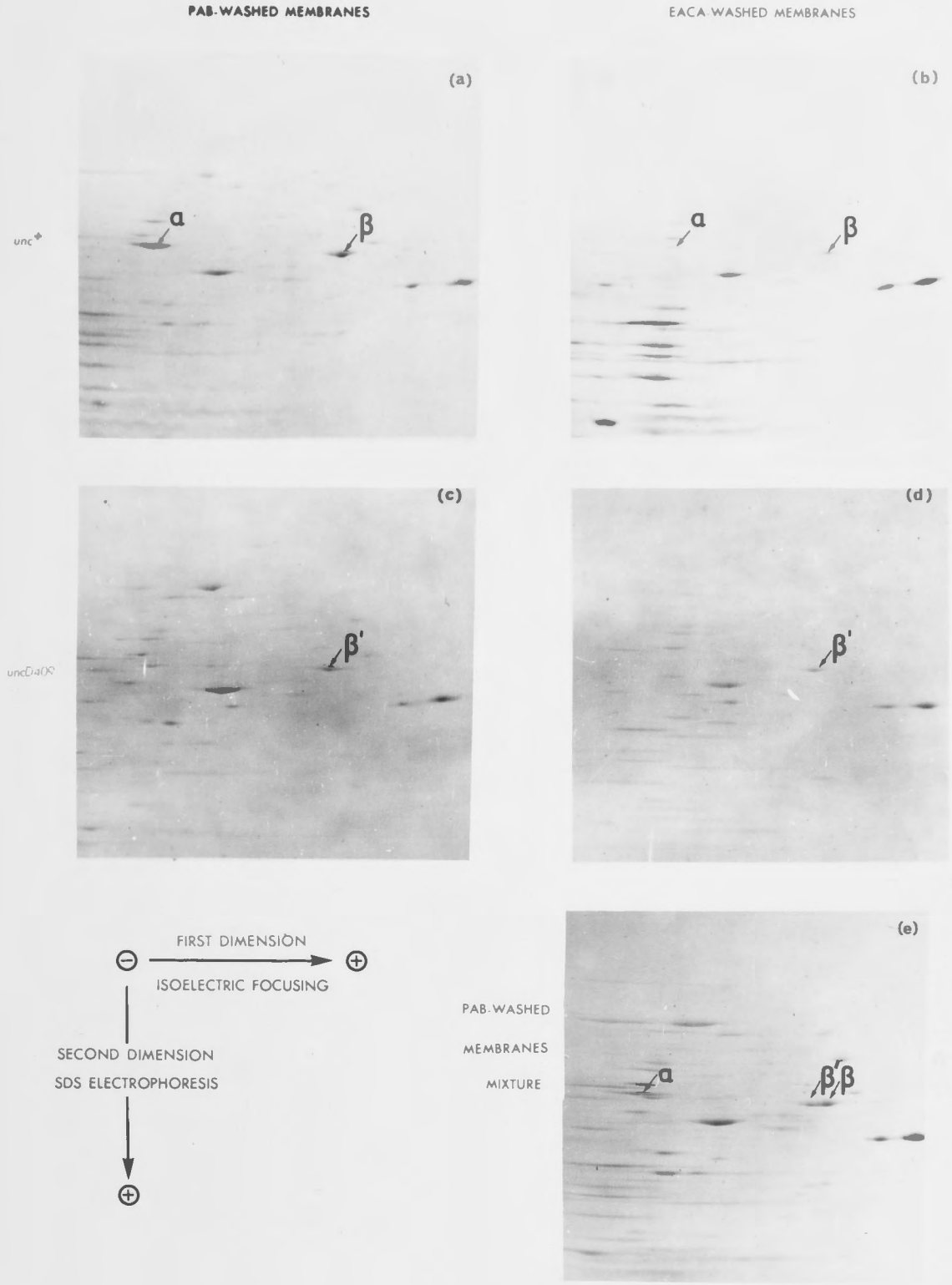


Figure VI.2. Two-dimensional analytical gel electrophoresis of washed membranes from strain AN248 (*unc* $^{+}$) and strain AN463 (*uncD409*). (a) PAB-washed membranes of strain AN248, 92µg of protein; (b) EACA-washed membranes of strain AN248, 134µg of protein; (c) PAB-washed membranes of strain AN463, 108µg of protein; (d) EACA-washed membranes of strain AN463, 135µg of protein; (e) mixture of PAB-washed membranes from strain AN248 (92µg of protein) and strain AN463 (123µg of protein). The normal α- and β-subunits of the Mg-ATPase and the abnormal β polypeptide are indicated by the arrows marked α, β and β' respectively. EACA-washed membranes were prepared as described in Scheme IV.1. The Ampholine composition used in the first dimension gels was 1:4 (pH ranges 3.5 to 10: 4 to 6), and the section of gel shown covers a pH gradient from about 6.5 to 4.8 (left to right). Details of the methods used are given in Chapter II. L and M.

normal β-subunits. A partial diploid strain, AN821 (*uncD409/unc*$^+$) (Cox *et al*, 1978a) was chosen for such an investigation. (The notation used indicates that the *uncD409* allele is on the plasmid, and the normal *unc* operon is on the chromosome).

The washed membranes from strain AN821 (*uncD409/unc*$^+$) were analysed by two-dimensional electrophoresis, to find out whether the normal and abnormal β-subunits were both produced by this strain. The PAB-washed membranes of the partial diploid strain contained both the normal and abnormal β-subunits of the Mg-ATPase (Fig.VI.3a). The normal β-subunit appeared to be present at a higher concentration than the abnormal β-subunit as judged by the relative sizes of the spots (Fig.VI.3a). Such as assessment is a reasonable indication of the amount of protein present (O'Farrell, 1975). In the EACA-washed membranes, the amount of the normal β-subunit was less than in the PAB-washed membranes, but the relative spot sizes of the normal and abnormal β-subunits had changed such that the abnormal β-subunit predominated (Fig.VI.3b).

The polypeptide composition of the EACA-wash obtained from the *uncD409/unc*$^+$ strain was investigated. Significantly, the abnormal β-subunit was present in the EACA wash, albeit at lower levels than the normal β-subunit (Fig.VI.3c), as would be expected from the reverse situation in the EACA-washed membranes. The Mg-ATPase preparation solubilized by chloroform treatment from PAB-washed membranes of AN821 (*uncD409/unc*$^+$) also contained normal and abnormal β-subunits (results not shown).

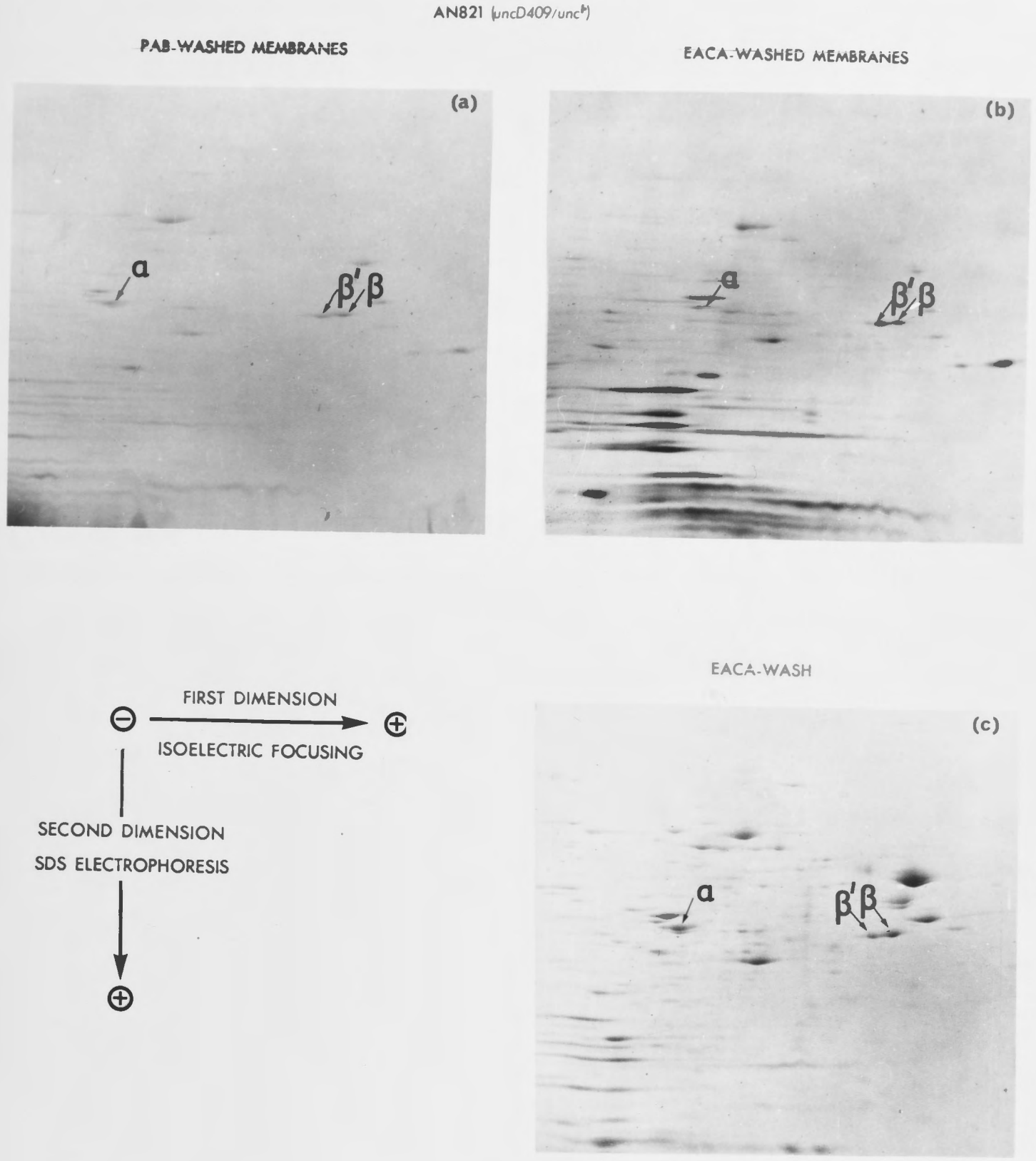


Figure VI.3. Two-dimensional analytical gel electrophoresis of PAB-washed membranes (a), EACA-washed membranes (b), and the concentrated EACA-wash (c) from the partial diploid strain AN 821 (*uncD409/unc*$^+$). Sample (a) contained 106µg of protein; sample (b) 144µg of protein; and sample (c) 101µg of protein. The normal α- and β-subunits of the Mg-ATPase and the abnormal β-subunit, are indicated by the arrows marked α, β, and β' respectively. EACA-washed membranes were prepared as described in Scheme IV.1. The Ampholine composition used in the first dimension gels was 1:4 (pH ranges 3.5 to 10: 4 to 6), and the pH gradient extended from about 6.5 to 4.8 (left to right). Details of the methods used are given in Chapter II.

Figure VI.4. Two-dimensional analytical gel electrophoresis of partially purified 5-subunit Mg-ATPase from the partial diploid strain AN821 (*uncD409/unc*$^{+}$). The EACA-wash (approximately 2mg of protein) was subjected to electrophoresis under non-dissociating conditions in a one-dimensional gel and the band containing Mg-ATPase activity was excised and applied to the analytical two-dimensional electrophoresis. The Ampholine composition used in the first dimension was 1:4 (pH ranges 3.5 to 10: 4 to 6), and the pH gradient extended from about 6.5 to 5.0 (left to right). Details of the methods used are given in Chapter II.

E. DETECTION OF THE ABNORMAL β-SUBUNIT IN Mg-ATPase AGGREGATES FROM THE PARTIAL DIPLOID STRAIN AN821 (*uncD409/unc*$^+$)

The concentrated EACA-wash (Fig.VI.3c) and the chloroform-solubilized preparation (gel not shown), from strain AN821 (*uncD409/unc*$^+$), contained the abnormal β-subunit in addition to the normal α- and β-subunits of the Mg-ATPase. In contrast, the EACA-wash from strain AN463 (*uncD409*) had barely detectable levels of the abnormal β-subunit, and none at all could be detected in the chloroform-solubilized preparation (results not shown). These results suggested that the abnormal β-subunit in the partial diploid strain had indeed assembled into an aggregate which could be solubilized in the normal way from the membranes.

The Mg-ATPase complex was partially purified from the EACA wash from strain AN821 (*uncD409/unc*$^+$) by slab gel electrophoresis in one dimension (Chapter II.K(b)), and a small amount of the altered β-subunit was found to co-migrate with the band of Mg-ATPase activity (Fig.VI.4). However, when the Mg-ATPase complex was purified by two-dimensional electrophoresis, very little of the abnormal β-subunit remained (results not shown).

F. DISCUSSION

The results presented above indicate that the *uncD409* mutation alters the β-subunit of the Mg-ATPase. Several lines of evidence support this conclusion. Firstly, PAB-washed membranes of strain AN463 (*uncD409*) lack the β-subunit of the Mg-ATPase found in PAB-washed membranes of a normal strain. There is however, a polypeptide present in membranes from

the mutant strain with a similar apparent molecular weight to the normal β-subunit, but with a slightly different isoelectric point. Secondly, this abnormal polypeptide is not found in PAB-washed membranes from either the normal strain AN248, or from a mutant strain (AN249) which lacks Mg-ATPase activity due to a mutation in the *uncA* gene (results not presented). If the lack of Mg-ATPase activity resulted in derepression of the synthesis of any normal membrane protein, that protein should be detectable in membranes of both the *uncA* and *uncD* mutants. Thirdly, PAB-washed membranes from the partial diploid strain AN821 (*uncD409/unc*$^+$) contain both the normal and abnormal β-subunits.

The experiments described in this chapter were undertaken on the basis of observations made by J. A. Downie. In these experiments (J. A. Downie, personal communication), the acridine dye atebrin was added to membrane fractions, and its fluorescence was monitored during the "energization" of the membranes by added NADH or ATP, and the "de-energization" by the respiratory inhibitor CN^- or the uncoupler CCCP. The quenching of atebrin fluorescence is observed during the "energization" of the membrane, and is considered to reflect the ability of the membranes to maintain a proton gradient (Deamer *et al*, 1972; Nieuwenhuis *et al*, 1973; Haddock and Downie, 1974). The ATP-induced atebrin fluorescence quenching depends on the presence of active Mg-ATPase bound to the 'F_0' site, as well as on the low permeability of the membrane to protons (Gibson *et al*, 1977a; Cox *et al*, 1978a; Haddock and Downie, 1974). Atebrin fluorescence quenching data obtained by J. A. Downie (personal communication) are presented in Fig.VI.5. The PAB-washed membranes from strain AN248 (*unc*$^+$), which retain the Mg-ATPase activity, exhibited both ATP- and NADH-induced quenching of atebrin fluorescence (Fig. VI.5a). In contrast, EACA-washed membranes, which had lost the Mg-ATPase, were permeable to protons, as

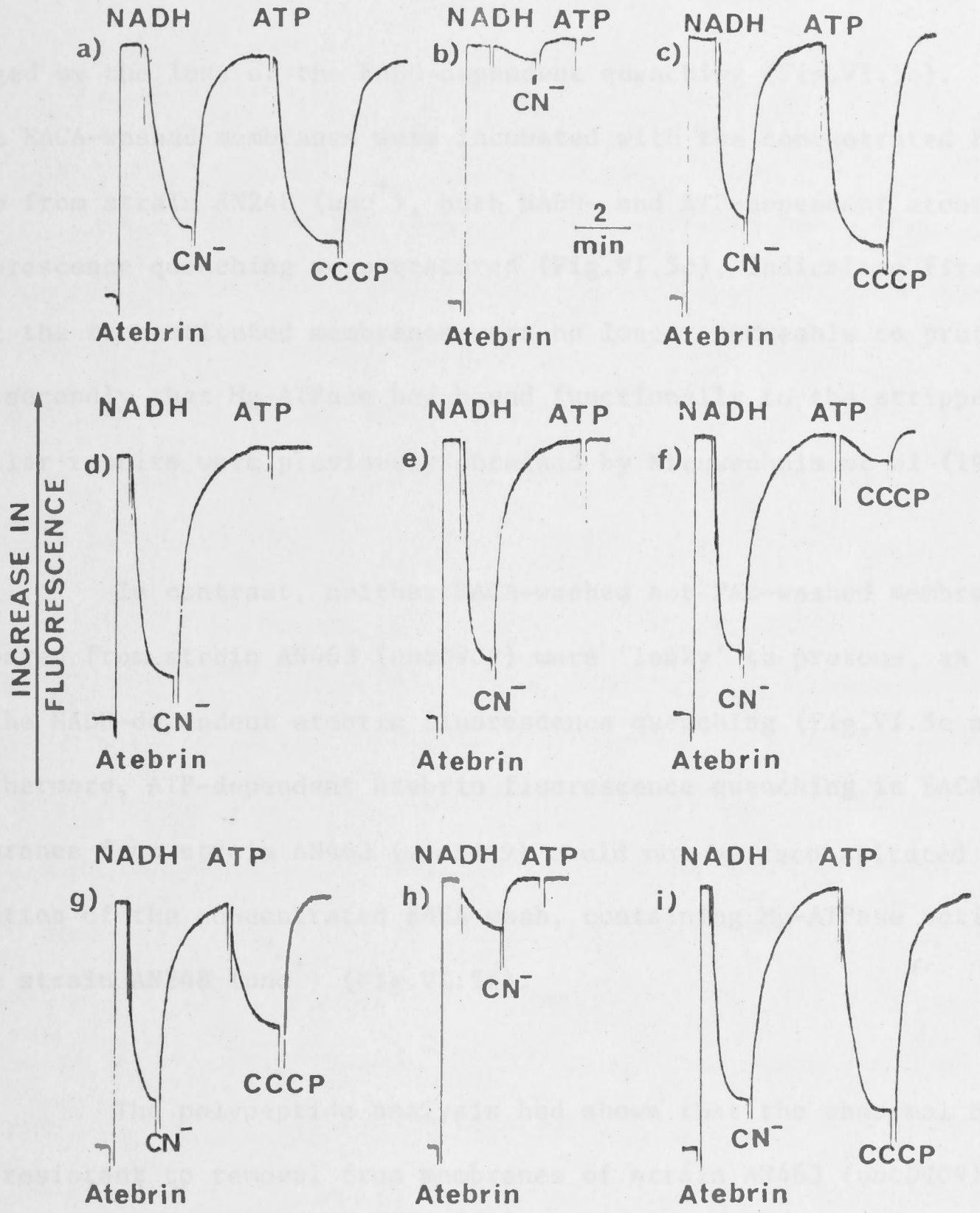


Figure VI.5 NADH- and ATP-induced atebrin fluorescence quenching in washed membranes from strain AN248 (*unc*$^+$) (a, b, c), strain AN463 (*uncD409*) (d, e, f) and the partial diploid strain AN821 (*uncD409/unc*$^+$ (g, h, i). PAB-washed membranes (a, d and g); EACA-washed membranes (b, e and h); EACA-washed membranes reconstituted with the EACA-wash from strain AN248 (*unc*$^+$) (c, f and i). Details of the reconstitution conditions and the measurement of atebrin fluorescence quenching are given in Chapter II.J. Atebrin was added to the diluted membranes to give a final concentration of 4μM, then NADH (2mM). After maximal quenching of fluorescence was obtained, cyanide (2.5mM) was added. ATP (1mM) was then added to the same incubation, and the degree of quenching was recorded. Carbonyl cynaide *m*-chlorophenylhydrazone (CCCP) was then added to 20μM (final concentration). These experimental results were kindly provided by Dr. J. A. Downie.

judged by the loss of the NADH-dependent quenching (Fig.VI.5b). When such EACA-washed membranes were incubated with the concentrated EACA-wash, also from strain AN248 (*unc*$^{+}$), both NADH- and ATP-dependent atebrin fluorescence quenching were restored (Fig.VI.5c), indicating firstly that the reconstituted membranes were no longer permeable to protons, and secondly that Mg-ATPase had bound functionally to the stripped membranes. Similar results were previously obtained by Nieuwenhuis *et al* (1973).

In contrast, neither EACA-washed nor PAB-washed membranes prepared from strain AN463 (*uncD409*) were 'leaky' to protons, as judged by the NADH-dependent atebrin fluorescence quenching (Fig.VI.5e and d). Furthermore, ATP-dependent atebrin fluorescence quenching in EACA-washed membranes from strain AN463 (*uncD409*) could not be reconstituted by the addition of the concentrated EACA-wash, containing Mg-ATPase activity, from strain AN248 (*unc*$^{+}$) (Fig.VI.5f).

The polypeptide analysis had shown that the abnormal β-subunit was resistant to removal from membranes of strain AN463 (*uncD409*) by low-ionic strength treatment in the absence of PAB, or by chloroform treatment. This observation clearly provided a structural explanation for the atebrin fluorescence quenching results.

The lack of proton permeability in EACA-washed membranes from strain AN463 (*uncD409*), as compared with similar membranes from the normal strain AN248, is presumably due to the retention on the membrane of the abnormal β-subunit. Furthermore, the lack of reconstitution of ATP-dependent fluorescence quenching when solubilized Mg-ATPase from a normal strain was added to EACA-washed membranes from strain AN463 (*uncD409*), suggests that the altered β-subunit is bound at sites normally occupied by the 5-subunit Mg-ATPase, so preventing functional binding of

the added Mg-ATPase.

A further defect found in the *uncD409* strain was the lack of an aggregate containing the normal α-subunit. Whether or not the minor subunits (γ, δ and ε) of the Mg-ATPase are bound with the altered β-subunit to the membrane has not yet been determined. However, it is apparent that the alteration in the β-subunit at least prevents normal assembly of the α-subunit into the Mg-ATPase.

In contrast, in the diploid strain AN821 (*uncD409/unc*$^+$) some membrane-bound Mg-ATPase aggregates were formed which appear to contain both abnormal and normal β-subunits. Since, as shown above, aggregates containing the abnormal β polypeptide do not assemble in the absence of the normal β-subunit, it is probable that the Mg-ATPase complexes of the diploid strain AN821 (*uncD409/unc*$^+$) must contain at least one normal β-subunit in order to assemble correctly. Such aggregates like those of a normal strain, are solubilized by low-ionic strength treatment in the absence of, but not in the presence of, PAB. The complexes containing the abnormal β-subunit appear to be selectively dissociated during isoelectric focusing.

It was apparent that some of the abnormal β-subunits were preferentially retained on the membranes of the diploid strain (*uncD409/unc*$^+$) during the washing procedure, suggesting that there was a class of abnormal β-subunits which were attached in the same way as in the *uncD409* strain, rather than assembled into complete aggregates.

Atebrin fluorescence quenching experiments (J. A. Downie, personal communication), together with previously published work (Cox

et al, 1978a) shed more light on the types of aggregate present in strain AN821 (*uncD409/unc*$^+$). When compared with the segregant strain (*unc* $^+$), membranes from the partial diploid strain (*uncD409/unc*$^+$) had a lower efficiency of ATP-induced atebrin fluorescence quenching, ATP-dependent transhydrogenase activity, and oxidative phosphorylation, and the Mg-ATPase activity of unwashed membranes was about 54% of that found in the segregant strain (Cox *et al*, 1978a). PAB-washed membranes from the diploid strain (Fig.VI.5g) retained the fluorescence quenching characteristics of the unwashed membranes (Cox *et al*, 1978a). The EACA-washed membranes from the diploid strain lost ATP-dependent atebrin fluorescence quenching (Fig.VI.5h), and the NADH-dependent quenching (Fig.VI.5h) was very much lower than that of strain AN463 (*uncD409*) (Fig.VI.5e).

Furthermore, normal reconstitution of both NADH- and ATP-induced atebrin fluorescence quenching occurs after the addition of the concentrated EACA-wash from strain AN248 (*unc*$^+$) to the EACA-washed membranes from the diploid strain (Fig.VI.5i). A similar reconstitution to that shown in Fig. VI.5i could also be achieved using the concentrated EACA-wash from the diploid strain instead of that from the normal strain. Thus membranes from the diploid strain AN821 (*uncD409/unc*$^+$) or the normal strain AN248 (*unc*$^+$) behave similarly when fractionated, except that PAB-washed membranes from the diploid strain have less efficient ATP-dependent atebrin fluorescence quenching.

The level of specific Mg-ATPase activity of the membranes from the *uncD409/unc*$^+$ strain is about 37% of the level one would expect to find in an *unc*$^+$*/unc*$^+$ partial diploid (Cox *et al*, 1978a; Gibson *et al*, 1977a, 1978) which would also suggest that a substantial number of the normal

β-subunits are in inactive complexes in the *uncD409/unc*$^{+}$ strain. Such 'inactivation' of the normal β-subunits may also explain the low efficiencies of ATP-dependent energization and oxidative phosphorylation mentioned above.

Chapter VII

DETECTION OF AN ABNORMAL β-SUBUNIT OF THE Mg-ATPase IN STRAINS CARRYING THE *unc-405* ALLELE

A. INTRODUCTION

The isolation and characterization of a strain carrying the *unc-405* allele was described by Cox *et al* (1974). The mutant allele was transferred by co-transduction with the *ilv* locus into strain AN248 (*ilvC*), to form the mutant strain AN285 (*ilv*$^+$, *unc-405*). The mutant strain had a low aerobic growth yield, characteristic of uncoupled strains, when grown on limiting concentrations of glucose.

Membranes from strains carrying the *unc-405* allele had very low Mg-ATPase activity, no detectable oxidative phosphorylation, no ATP-dependent transhydrogenase activity, and no ATP-induced quenching of atebrin fluorescence (Cox *et al*, 1974; Gibson *et al*, 1977a). Moreover, no inactive aggregate corresponding to the Mg-ATPase could be found in the low-ionic strength wash from membranes of strain AN285 (*unc-405*) (Cox *et al*, 1974). In these respects, therefore, membranes from strain AN285 (*unc-405*) resembled those from the *uncD409* mutant strain AN463, described in Chapter VI.

However, the membranes from these two strains (*uncD409* and *unc-405*) were different in several important respects. Firstly, ATP-dependent transhydrogenase activity, or ATP-induced atebrin fluorescence quenching, could be partially reconstituted in unwashed membranes from strain AN285 (*unc-405*) by the addition of solubilized Mg-ATPase from a normal strain (Cox *et al*, 1974; Gibson *et al*, 1977b; J. A. Downie,

unpublished work). In contrast, neither of these activities could be restored by the addition of Mg-ATPase to membranes from strain AN463 (*uncD409*), even if these membranes had first been subjected to the EACA-washing procedure (see Chapter VI). Secondly, both the washed and unwashed membranes from the *unc-405* strain had NADH-induced atebrin fluorescence quenching, but the level of this quenching could be increased by the addition of DCCD (dicyclohexyl-carbodi-imide) to the membranes (J. A. Downie, unpublished results). This observation indicated that the membranes from the *unc-405* strain had abnormally high proton permeability, since DCCD has been shown previously to decrease the proton permeability of stripped membrane particles from *E.coli* (Rosen, 1973b; Altendorf *et al*, 1974; Patel and Kaback, 1976). In contrast, the membranes from strain AN463 (*uncD409*) retained essentially maximal NADH-induced atebrin fluorescence quenching after the EACA-washing procedure (see Chapter VI). It should be noted that the effect of DCCD in the *unc-405* strain was sometimes masked by the high levels of NADH-induced quenching associated with elevated NADH-oxidase rates (c.f. Gibson *et al*, 1977a).

Recently, plasmids have been obtained carrying *unc* alleles in the *unc*$^{+}$, *uncA*, *uncB*, *uncC* and *uncD* genes (Gibson *et al*, 1977b; Cox *et al*, 1978a). Each plasmid was introduced into a recombination-deficient strain carrying the *unc-405* allele, and the resulting diploid strains could grow on succinate only if genetic complementation had occurred. The plasmids carrying the *unc*$^{+}$, *uncB402*, or *uncC424* alleles complemented the *unc-405* mutation. The plasmid carrying the *uncA401* allele complemented the *unc-405* mutation very poorly. The strain carrying both the *uncD409* and *unc-405* alleles, in contrast, did not grow at all (Gibson *et al*, 1977a; F. Gibson, unpublished results). These

results indicated that the *unc-405* mutation was probably in the *uncD* gene.

Since the *uncD409* allele had been shown to affect the β-subunit of the Mg-ATPase (see Chapter VI), and it was likely that the *unc-405* allele was in the *uncD* gene, it was possible that there was a detectable alteration in the β-subunit in the *unc-405* mutant strain. Moreover, since the *unc-405* and *uncD409* mutations affected the proton permeability of the membrane, but in quite different ways, it was clear that an investigation into the nature of the lesion in the *unc-405* strain might shed some light on the relationship between the proton 'channel' and the components of the Mg-ATPase. The membranes from strain AN285 (*unc-405*) were therefore analysed by electrophoresis, and the results reported below.

B. POLYPEPTIDE PROFILES OF WASHED MEMBRANES FROM STRAIN AN285 (*unc-405*)

The polypeptides of the PAB-washed membranes from strain AN285 (*unc-405*) did not include the normal β-subunit, and there were only faint traces of the α-subunit remaining (Fig.VII.1a). The PAB-washed membranes from the normal strain AN248 are shown for comparison (Fig.VII.1b). However, there was a polypeptide present in the membranes of the mutant strain (Fig.VII.1a) , but not in those of the normal strain (Fig.VII.1b), which had the same apparent molecular weight as the normal β-subunit, but a more acidic isoelectric point. The difference between the isoelectric points was confirmed by the electrophoresis of a mixture of PAB-washed membranes from strains AN285 (*unc-405*) and AN248 (unc^+) (Fig.VII.1c). The abnormal β-subunit was also present in the EACA-washed membranes from strain AN285 (*unc-405*) (Fig.VII.1d).

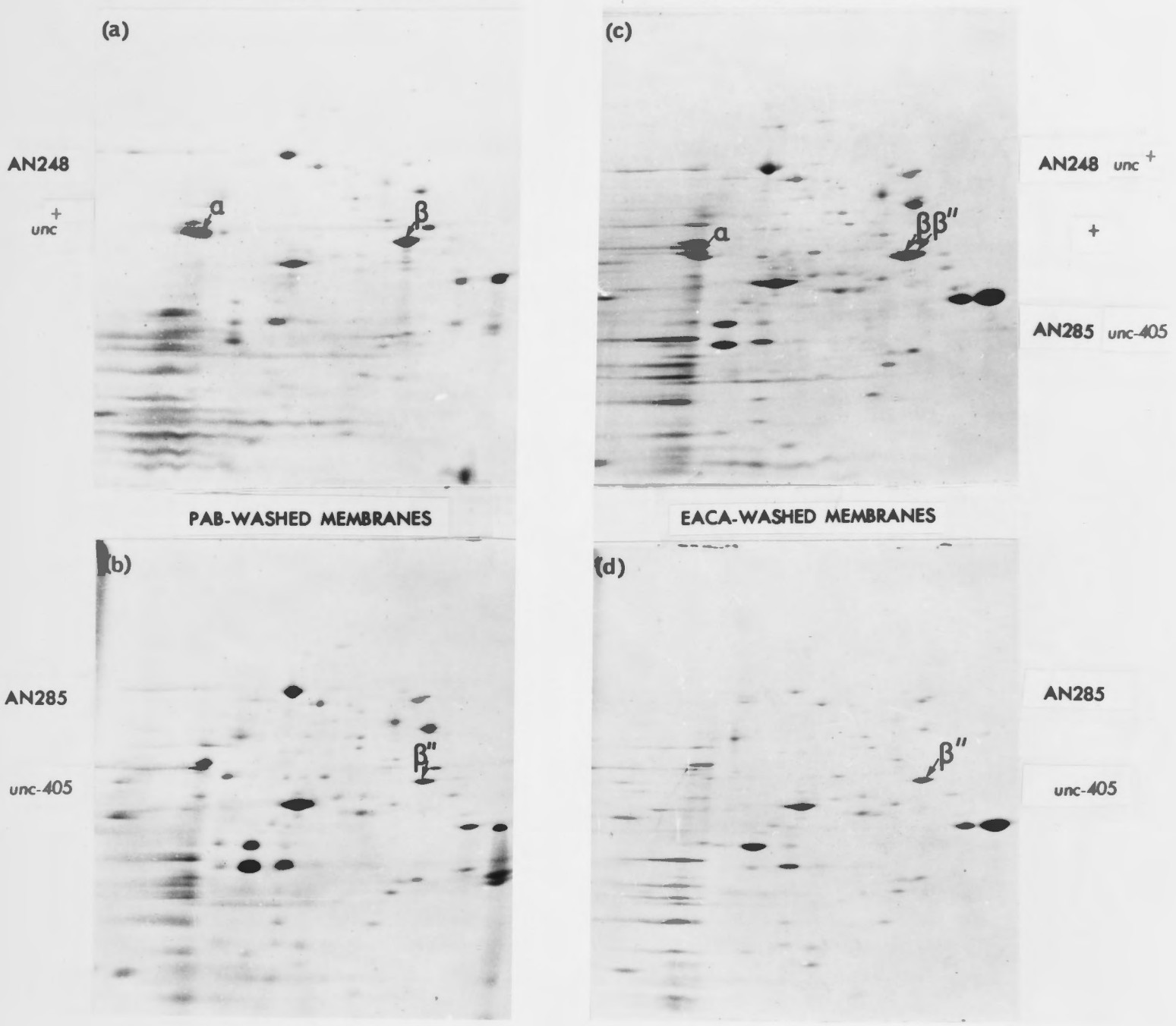


Figure VII.1. Two-dimensional analytical gel electrophoresis of washed membranes from strain AN248 (*unc*$^+$) and strain AN285 (*uncD405*). (a) PAB-washed membranes from strain AN248 (*unc*$^+$), 196µg of protein; (b) PAB-washed membranes from strain AN285 (*uncD405*), 197µg of protein; (c) mixture of PAB-washed membranes from strain AN248 (64µg of protein) and strain AN285 (114µg of protein); (d) EACA-washed membranes from strain AN285 (*uncD405*), 146µg of protein. The normal α- and β-subunits of the Mg-ATPase and the abnormal β polypeptide are indicated by the arrows marked α, β and β" respectively. The EACA-washed membranes were prepared as described in Scheme IV.1. The Ampholine composition used in the first dimension gels was 1:1:3 (pH ranges 3.5 to 10: 5 to 7: 4 to 6). The section of gel shown encompasses a pH range similar to that shown in Fig. IV.2a. Details of the methods used are given in Chapter II.

Since both the *uncD409* and the *unc-405* alleles alter the β-subunit of the Mg-ATPase, the *unc-405* mutation affects the *uncD* gene and is therefore termed *uncD405*.

C. DETECTION OF THE NORMAL AND ABNORMAL β-SUBUNITS OF THE Mg-ATPase IN MEMBRANES FROM THE PARTIAL DIPLOID STRAIN AN785 (*uncA401/uncD405*)

Amongst the stock of strains available in the laboratory, there was a partial diploid strain AN785 (*uncA401/uncD405*), constructed as described by Gibson *et al* (1977b), which might be expected to contain both the abnormal β-subunit, produced by the *uncD405* operon on the chromosome, as well as the normal β-subunit, produced by the *uncA401* operon on the plasmid. The *uncA401* mutation would not be expected to affect the β-subunit of the Mg-ATPase, since this mutation is not in the *uncD* gene (Cox *et al*, 1978a; see also Chapter VI).

The membranes from strain AN785 (*uncA401/uncD405*) were therefore analysed, to ascertain whether both β polypeptides were present. The pattern obtained after two-dimensional gel electrophoresis of the unwashed membranes from strain AN785 (*uncA401/uncD405*) is shown in Fig. VII.2a. Both the abnormal (*uncD405*) and normal (*uncA401*) β-subunits are present, and are denoted β" and β respectively (Fig.VII.2a). The membranes from strain AN785 (*uncA401/uncD405*) were also fractionated using the Minus-PAB washing procedure (Scheme IV.2b). The concentrated Minus-PAB wash contained both the normal and abnormal β-subunits, in approximately equal amounts as judged by their spot size (Fig.VII.2b). This parameter is a reasonable indication of the amount of protein present

Figure VII.2 Two-dimensional analytical gel electrophoresis, over the acidic pH range, of the unwashed membranes (a), the concentrated Minus-PAB wash (b), and the Mg-ATPase purified from the Minus-PAB wash (c), all from the partial diploid strain AN785 (*uncA401/uncD405*). The α-, δ- and ε-subunits, as well as the normal and abnormal β-subunits (labelled β and β" respectively), are indicated. Sample (a) contained 151μg of protein, and sample (b), 153μg of protein. The Mg-ATPase (b) was purified by two-dimensional electrophoresis, starting with 1.3mg of protein of the Minus-PAB wash from the diploid strain, and using a small amount of the chloroform-solubilized Mg-ATPase preparation from strain AN248 (*unc*$^{+}$) as a marker (about 5% of the amount used in the gels of Fig.V.11). The Ampholine composition used in the first dimension gels was 1:1:3 (pH ranges 3.5 to 10: 5 to 7: 4 to 6). The pH gradient may be compared with that shown in Fig.V.7. Details of the methods are given in Chapter II.

(O'Farrell, 1975).

D. DETECTION OF THE ABNORMAL β-SUBUNIT IN Mg-ATPase AGGREGATES SOLUBILIZED FROM THE MEMBRANES OF THE PARTIAL DIPLOID STRAIN AN785 (*uncA401/uncD405*)

Haploid strains carrying the *uncA401* allele had been shown to contain inactive Mg-ATPase aggregates otherwise very similar to the normal Mg-ATPase (Cox *et al*, 1974; Maeda *et al*, 1976; Bragg and Hou, 1977). The partial diploid strain AN785 (*uncA401/uncD405*) would therefore be expected to produce, as well as some normal Mg-ATPase, Mg-ATPase aggregates of the *uncA401* type. It was of interest to see whether there was also a population of Mg-ATPase aggregates containing the abnormal β-subunit, since this polypeptide had been found in the Minus-PAB wash (Fig.VII.2b).

The majority of the Mg-ATPase aggregates in this strain should contain one or more copies of the subunit produced by the *uncA401* allele which would probably render such aggregates inactive. In addition, if the abnormal (*uncD405*) β-subunits were also distributed amongst these complexes, one would predict that only a small number of active Mg-ATPase proteins would be present. In anticipation of the low Mg-ATPase activity expected, therefore, the concentrated Minus-PAB wash from strain AN785 (*uncA401/uncD405*) was subjected to the two-dimensional purification procedure together with a small amount of the chloroform-solubilized preparation from strain AN248 (unc^+). Following electrophoresis, the section of gel containing Mg-ATPase activity was excised and subjected to two-dimensional analytical gel electrophoresis. The normal (β) and

abnormal (β") polypeptides were present in the purified complex in comparable amounts, and the α-, δ- and ε-subunits (Fig.VII.2c) and the γ-subunit, which cannot be seen on this gel because of the acidic pH range used, were also present. The contribution of the subunits of the chloroform-solubilized Mg-ATPase from strain AN248 (*unc*$^+$) to the pattern shown in Fig.VII.2c. was considered to be negligible, since the amount present was only about 5% of the amount applied to the gel of Fig.V.11. In the gel used to purify the Mg-ATPase complex from strain AN785 (*uncA401/uncD405*), the shape of the spot containing Mg-ATPase indicated that the complexes from the diploid strain might have significant activity. The Mg-ATPase activities of the unwashed membranes and the Minus-PAB wash from the diploid strain were therefore compared with the activities in the normal strain AN248 (*unc*$^+$). The Mg-ATPase activity present in unwashed membranes from strain AN785 (*uncA401/uncD405*) was about 13% of that in membranes from strain AN248 (*unc*$^+$), and the activity recovered in the Minus-PAB wash from the diploid strain was about 7% of that recovered in the Minus-PAB wash from the normal strain (Table VII.1).

In the unwashed membranes from strain AN785 (*uncA401/uncD405*), the level of NADH-induced atebrin fluorescence quenching (50%) was lower than that of normal membranes, whilst the level of ATP-induced quenching in the membranes from the diploid strain was very low (about 5%) (trace not shown).

E. DETECTION OF TWO ABNORMAL β-SUBUNITS IN MEMBRANES FROM THE PARTIAL DIPLOID STRAIN AN834 (*uncD409/uncD405*)

The separation between the abnormal (*uncD405*) β-subunit and

TABLE VII.1 Mg-ATPase Activities in Fractions from Strain AN785 (*uncA401/uncD405*) and Strain AN248 (*unc*$^+$)

	Fraction	AN785 *uncA401/uncD405* a	AN248 *unc*$^+$ b	$\frac{a}{b}$ x 100
SPECIFIC ACTIVITIES (units*/ mg protein)	Unwashed Membranes	0.10	0.74	13
	Minus-PAB wash	0.09	1.3 #	7
TOTAL ACTIVITY (units*/g wet weight of cells)	Minus-PAB wash	0.5	5.9	8

* Units = μmoles.min^{-1}

\# The specific activity of the Minus-PAB wash was lower than that reported in Chapter III.C. The present preparation was made using shorter ultracentrifugation times than those described in the method (Chapter II.H), and there was incomplete separation of membrane particles from the soluble fraction. For this reason, the table includes figures for the total activity recovered in the Minus-PAB washes per g wet weight of washed cells.

the normal β-subunit on gels was not very great, and it was important to eliminate the unlikely possibility that all of the above results were due to some charge modification unrelated to the mutation. The partial diploid strain AN834 (*uncD409/uncD405*), was constructed by the method of Gibson *et al* (1977b), and was obtained from the stock of strains in the laboratory. This strain might be expected to produce widely-separated abnormal β-subunits, but no normal β-subunit. It was also of interest to ascertain whether aggregates containing the α-subunit could assemble in the absence of normal β-subunits. Therefore the polypeptides of membranes from strain AN834 (*uncD409/uncD405*) were analysed by two-dimensional electrophoresis.

Both of the abnormal β polypeptides were present in gel profiles of the unwashed membranes (Fig.VII.3a), the PAB-washed membranes (Fig.VII.3b), and the EACA-washed membranes (Fig.VII.3c) of strain AN834 (*uncD409/uncD405*). No normal β-subunit was present, and the α-subunit was readily lost during PAB-washing of the membranes (Fig.VII.3a and b). There is no detectable change in the relative intensities of the two abnormal polypeptides during the complete washing procedure, although it is apparent that the *uncD405* polypeptide (labelled β") is present in lower amounts than the *uncD409* polypeptide (labelled β') as judged by the relative sizes of the spots, and their relative staining intensities.

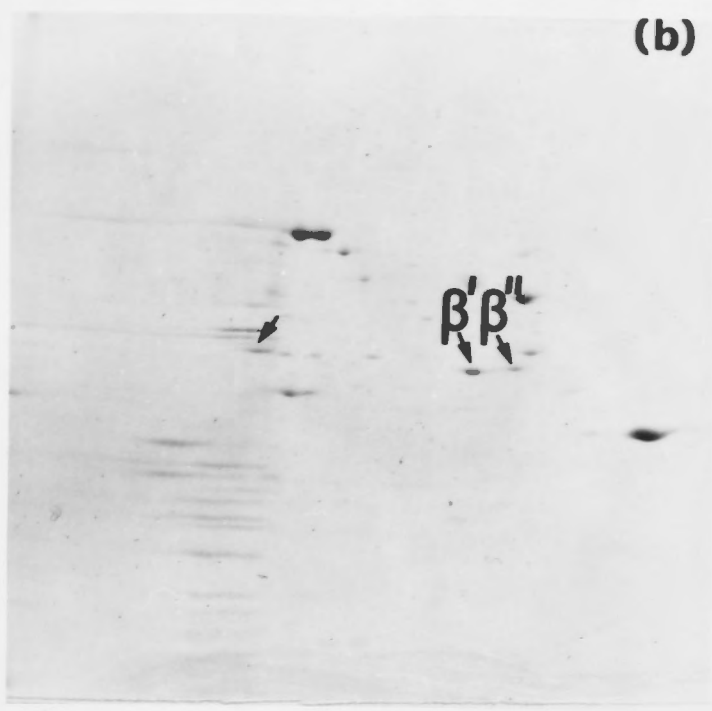
F. FURTHER INVESTIGATIONS INTO THE NATURE OF RESIDUAL Mg-ATPase AGGREGATES IN THE MEMBRANES OF STRAIN AN834 (*uncD409/uncD405*)

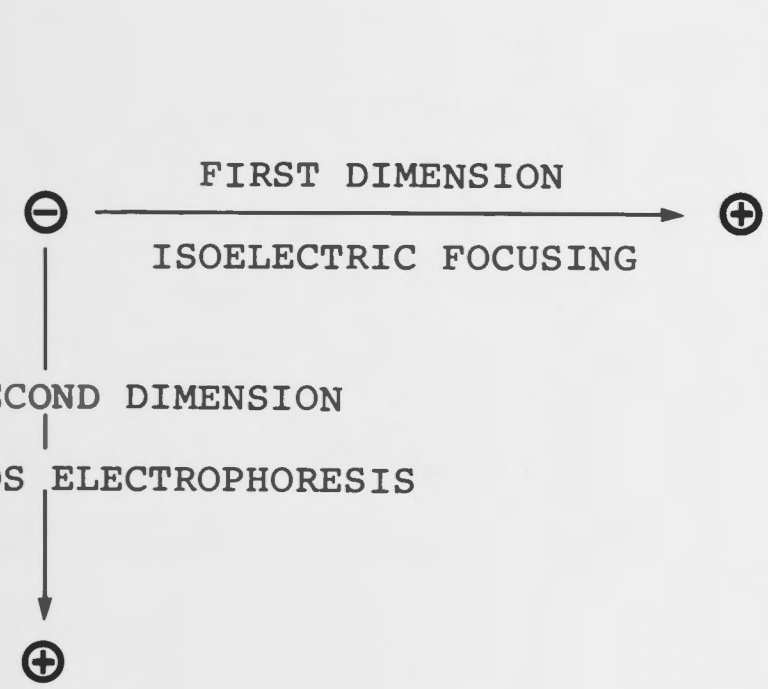
It was apparent from the above results that the α-subunit was probably not bound to the membranes, whereas both abnormal β polypeptides

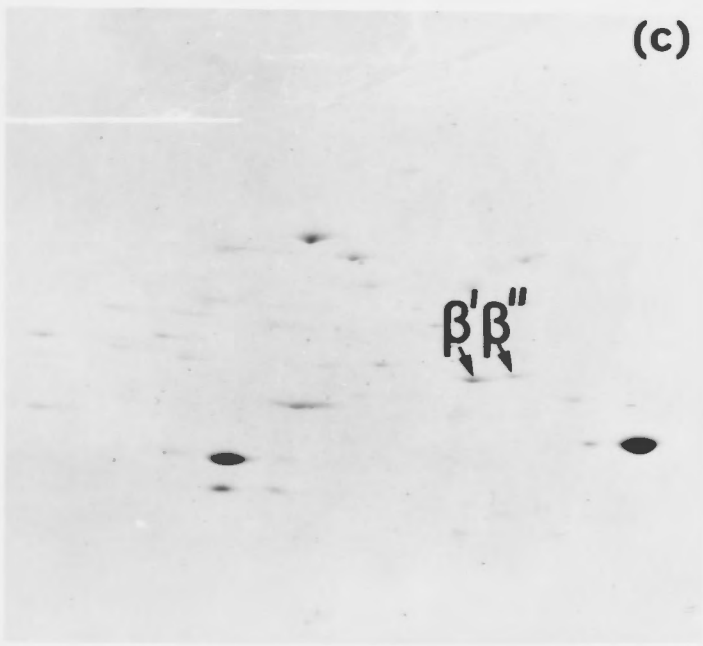
AN834 (*uncD409/uncD405*)

UNWASHED MEMBRANES

PAB-WASHED MEMBRANES







EACA-WASHED MEMBRANES

Figure VII.3. Two-dimensional analytical gel electrophoresis of membranes from the partial diploid strain AN834 (*uncD409/uncD405*). (a) unwashed membranes, 152μg of protein; (b) PAB-washed membranes, 160μg of protein; (c) EACA-washed membranes, 139μg of protein. The normal α-subunit of the Mg-ATPase, and the abnormal *uncD409* and *uncD405* β polypeptides, are indicated by the arrows marked α, β' and β'' respectively. The EACA-washed membranes were prepared as described in Scheme IV.2b. The Ampholine composition of the first dimension gel was 1:1:3 (pH ranges 3.5 to 10: 5 to 7: 4 to 6). The section of gel shown encompasses a pH range similar to that shown in Fig. IV.2a. Details of the methods used are given in Chapter II.

remained bound to the membranes throughout the EACA-washing procedure. It was therefore likely that these membranes contained membrane complexes both of the *uncD405* type (F_0-β") and of the *uncD409* type (F_0-β'). The following atebrin fluorescence quenching data (Fig. VII.4) suggested that the former type of complex was present. The unwashed membranes from strain AN834 (*uncD409/uncD405*) had no ATP-induced atebrin fluorescence quenching, and the NADH-induced quenching (37%) was lower than that of strain AN463 (*uncD409*) or strain AN248 (*unc*$^+$) (cf. Fig.VI.1). The degree of NADH-induced quenching could be increased by the addition of the Minus-PAB wash from strain AN248 (*unc*$^+$), and ATP-dependent quenching was also reconstituted to some extent (Fig. VII.4b).

As expected from the above results, membranes from strain AN834 (*uncD409/uncD405*) lacked ATP-dependent transhydrogenase activity and had very low P/O ratios (J. Radik, unpublished results) and no Mg-ATPase activity (results not shown).

G. DISCUSSION

The results presented indicate that the *unc-405* mutation alters the β-subunit of the Mg-ATPase. Firstly, PAB-washed membranes from strain AN285 (*unc-405*) do not contain the normal β-subunit of the Mg-ATPase, but there is a polypeptide present in the gel profiles, which has the same apparent molecular weight as the normal β-subunit, but a different apparent isoelectric point. Secondly, this polypeptide is not present in the membranes of a normal strain. Thirdly, membranes from the partial diploid strain AN785 (*uncA401/uncD405*) have the abnormal β-subunit ascribed to the *uncD405* mutation, as well as the normal β-subunit.

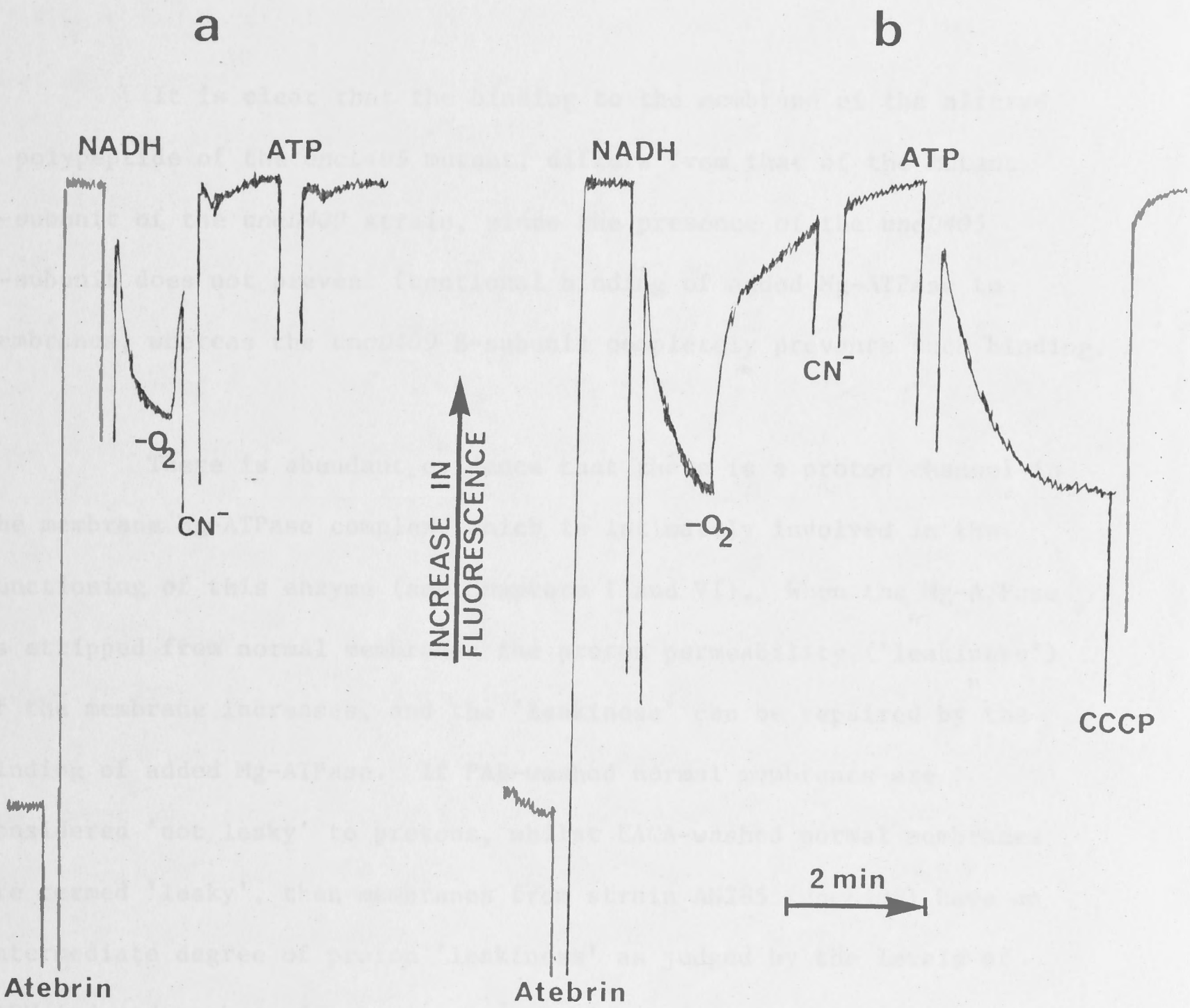


Figure VII.4. NADH- and ATP-induced atebrin fluorescence quenching in unwashed membranes from the partial diploid strain AN834 (*uncD409/uncD405*) without added Mg-ATPase (a), and reconstituted with the minus PAB wash from strain AN248 (*unc*$^+$) (b). Details of the reconstitution conditions and the measurement of atebrin fluorescence quenching are given in Chapter II.J. Atebrin was added to the diluted membranes to give a final concentration of 2μM, then NADH to 1mM. Cyanide (2.5mM), ATP (0.8mM) and CCCP (4μM; (b) only) were then added in sequence. The NADH oxidase rate was sufficiently high to exhaust the available O_2 at the times indicated.

Fourthly, membranes from the partial diploid strain AN834 (*uncD409/uncD405*) contain the abnormal β polypeptide produced by the *uncD409* allele, and also the abnormal β polypeptide attributable to the *uncD405* allele, but not the normal β-subunit of the Mg-ATPase.

It is clear that the binding to the membrane of the altered β polypeptide of the *uncD405* mutant, differs from that of the mutant β-subunit of the *uncD409* strain, since the presence of the *uncD405* β-subunit does not prevent functional binding of added Mg-ATPase to membranes, whereas the *uncD409* β-subunit completely prevents such binding.

There is abundant evidence that there is a proton channel in the membrane Mg-ATPase complex, which is intimately involved in the functioning of this enzyme (see Chapters I and VI). When the Mg-ATPase is stripped from normal membranes the proton permeability ('leakiness') of the membrane increases, and the 'leakiness' can be repaired by the binding of added Mg-ATPase. If PAB-washed normal membranes are considered 'not leaky' to protons, whilst EACA-washed normal membranes are termed 'leaky', then membranes from strain AN285 (*uncD405*) have an intermediate degree of proton 'leakiness' as judged by the levels of NADH-induced atebrin fluorescence quenching. A further indication that the proton permeability of the *uncD405* mutant membranes is abnormal, is the increase in NADH-induced-atebrin fluorescence quenching observed following the addition of DCCD to the PAB-washed membranes from strain AN285 (*uncD405*).

It is thus possible that these membranes are actually 'leaky', but that the high NADH oxidase rates in this strain are able to maintain a proton gradient across the membrane. The 'intermediate' degree of

proton-leakiness could alternatively be due to the presence of the abnormal β polypeptides, bound to the 'F_0' sector in such a way that all the proton channels are partially blocked. The reconstitution of ATP-driven energization of the membrane after the addition of normal Mg-ATPase would then be difficult to explain, unless it was assumed that the abnormal β polypeptide could be easily displaced, in which case full reconstitution should be possible. Full restoration of ATP-driven atebrin fluorescence quenching was not observed. In any case, this situation differs from that in membranes of strain AN463 (*uncD409*), in which the presence of the *uncD409* β-subunit completely blocks the proton channel, as well as the Mg-ATPase binding sites, even after the EACA-washing of the membranes.

However, an alternative explanation for the reconstitution data is also possible. A comparison of the polypeptides of PAB-washed membranes from strains AN285 (*uncD405*) and AN248 (*unc*$^+$) indicates that the relative amount of the *uncD405* β polypeptide present is less than the amount of β-subunit found in normal membranes. Also, the amount of the *uncD405* β-subunit appears to be considerably less than the amount of the *uncD409* β-subunit in the membranes from the diploid strain AN834 (*uncD409/uncD405*). Similarly, the normal β-subunit predominates in the membranes from strain AN785 (*uncA401/uncD405*). These results would suggest that the strains carrying the *uncD405* mutation either produce the abnormal β polypeptide in reduced amounts, or produce a β polypeptide which is susceptible to degradation. If the other products of the *unc* operon were produced in normal amounts, the amounts of *uncD405* β polypeptide available would be insufficient to saturate the available Mg-ATPase binding sites, and the corresponding proton channels would also be exposed. On the other hand, the amounts of the *uncD409* β polypeptide in strains carrying this allele are sufficient to saturate

such sites.

Rosenberg *et al* (1975) found that the aerobic uptake of serine and of P_i by whole cells of strain AN285 (*uncD405*) was identical with that found in cells of the normal strain AN248. Having assumed that the Mg-ATPase was completely absent, these workers deduced that the presence of the Mg-ATPase aggregate was not necessary for transport. However, the present work would suggest that this conclusion was premature. If only a portion of the F_0 sites are occupied by the residual β polypeptide in the mutant strain, then the observed transport may simply have been supported by abnormally high oxidase rates, and thus comparison with the normal strain would be precluded. If, on the other hand, all the F_0 sites are occupied by abnormal β polypeptides, then this would suggest that they could possibly have a structural role in the maintenance of membrane energization.

The partial diploid strain AN785 (uncD401/uncD405) produced some Mg-ATPase aggregates containing the abnormal (uncD405) β-subunit. Overall, the stoichiometry of the normal and abnormal β-subunits appeared to be approximately 1:1. Presumably, therefore, the majority of the individual complexes contained both normal and abnormal β-subunits. It is unlikely that all the β-subunits of any single ATPase-like aggregate were abnormal, since such a complex was not found in the haploid uncD405 strain. The number of β-subunits present per Mg-ATPase complex is not yet known (see Chapter X).

It would appear that there are a few uncD405 type complexes present in membranes from the uncD401/uncD405 strain, since the efficiency of

NADH-induced atebrin fluorescence quenching in this strain was somewhat lower than in a normal strain. The effect of DCCD was not tested. It is also clear that a small proportion of the hybrid Mg-ATPase complexes in this diploid strain are active, since the Mg-ATPase activity is low but not negligible, and there appears to be a small amount of ATP-driven fluorescence quenching, despite the 'leakiness' of the membranes mentioned above. Such molecules may be completely normal, but it is conceivable that hybrid molecules containing one or more altered subunits could also have low levels of activity.

Strain AN785 (*uncA401/uncD405*) grows very poorly on succinate. The growth yield on limiting concentrations of glucose was not measured, but the growth yield of a strain with similar properties, AN798 (*uncD405/uncA401*) was 120 Klett units, or 170 Klett units in the presence of added Luria broth (F. Gibson, unpublished results). The former value is the same as that obtained for a haploid strain carrying the *uncD405* allele (Gibson *et al*, 1977a), but the latter value indicates that some *in vivo* complementation has occurred (see Cox *et al*, 1978a). Together with the observation that strain AN785 (*uncA401/uncD405*) grows, albeit poorly, on succinate, these data would suggest that the degree of *in vivo* complementation, which would correlate with the formation of active Mg-ATPase (F_1-F_0) complexes, is somewhat dependent on growth conditions.

It is clear that the proportion of Mg-ATPase aggregates containing the abnormal (*uncD405*) β-subunit in strain AN785 (*uncA401/uncD405*) is greater than the proportion containing the *uncD409* β-subunit in strain AN821 (*uncD409/unc*$^+$) (Chapter VI), especially after the respective purification procedures. This would indicate that the *uncD405* β-subunit is more amenable than the *uncD409* β-subunit to stable incorporation into an aggregate in the presence of normal β-subunits.

The alteration caused by the *uncA401* allele has not yet been identified, either by gel electrophoresis (results not presented) of fractions from strain AN249 (*uncA401*) (Cox *et al*, 1973b) or strain AN785 (*uncA401/uncD405*), or by work done in other laboratories. When washed in low-ionic strength buffer, the membranes from the *uncA401* strain are indistinguishable from normal washed membranes, as shown by the reconstitution of ATP-driven transhydrogenase activity by a normal Mg-ATPase preparation (Cox *et al*, 1973a,b). The inactive Mg-ATPase complex from an *uncA401* strain was of a similar size and composition to the normal Mg-ATPase (Cox *et al*, 1974). The 5 types of subunits of the *uncA401* complex are of similar size to those of the normal Mg-ATPase, and the amounts of tightly-bound nucleotides in both complexes were also similar (Maeda *et al*, 1976). The 5-subunit *uncA401* complex has recently been purified and characterized by Bragg and Hou (1977). These workers found that when the normal or the mutant complexes were treated with a cross-linking reagent, similar products were obtained, indicating that the complexes were organized in the same way. They also found that the mutant complex could bind to urea-treated membranes, restoring electron-transport dependent transhydrogenase activity. J. A. Downie (personal communication) has found that the Mg-ATPase from an *uncA401* mutant could restore proton impermeability to stripped membranes. Both these observations indicate that the inactive complex binds normally to the 'F_0 sector'. The only difference so far noted between the *uncA401* and *unc*$^+$ Mg-ATPases, aside from the presence or absence of Mg-ATPase activity, was reported by Bragg and Hou (1977), who found that the mutant complex had a slightly-increased affinity for exogenously-added ADP.

The diploid strain AN834 (*uncD409/uncD405*) appears to have

no aggregates containing the α-subunit, as judged by the lack of the α-subunit in washed membranes. The nature of the residual complexes was suggested by atebrin fluorescence quenching and gel electrophoresis experiments. The absence of full reconstitution of ATP-dependent and NADH-dependent quenching is consistent with the presence of complexes of the *uncD405* type, since the membranes from strain AN285 (*uncD405*) showed similar behaviour. The presence of complexes of the *uncD409* type could be inferred from the behaviour of the *uncD409* β polypeptide during the washing of the membranes. The presence of such complexes in the membrane would not be apparent during atebrin fluorescence quenching experiments, since the number of F_0-sector proteins attributable to the plasmid would equal the number of the *uncD409* β-subunits, and the remaining F_0-sector proteins would be the only ones with detectable 'activity', in terms of either Mg-ATPase binding, or proton permeability.

The aerobic growth yield of strain AN834 (*uncD409/uncD405*) on 5mM-glucose was 99 Klett units, or if 5% Luria broth was included, 134 Klett units. These values are similar to those found for a haploid strain carrying the *uncD409* allele (108 and 137 Klett units respectively; Cox *et al*, 1978a). The *uncD409* allele may have a more detrimental effect on growth yield than the *uncD405* allele (cf. Cox *et al*, 1978a and Gibson *et al*, 1977a).

Chapter VIII

ISOLATION AND CHARACTERIZATION OF MUTANT STRAINS CARRYING THE *unc-436* OR *unc-441* ALLELES

A. INTRODUCTION

Mutations directly affecting the membrane Mg-ATPase complex (F_1-F_0) have either been located in the *unc* operon (Cox and Gibson, 1974; Gibson *et al*, 1978), or in the same region of the chromosome (see Haddock and Jones, 1977), at about min 83 on the revised chromosome map of *E.coli* (Bachmann *et al*, 1976). This region of the chromosome is co-transducible with the *ilvC* gene (Butlin *et al*, 1971).

A 'localized' mutagenesis technique was therefore used to isolate, by co-transduction with *ilvC*, mutants which could not grow on minimal medium supplemented with succinate, but could grow with glucose as the carbon source.

B. 'LOCALIZED' MUTAGENESIS

The localized mutagenesis procedure, described in Chapter II, F., was developed by Murgola and Yanofsky (1974) from the method of Hong and Ames (1971), and has also been used by Simoni and Shandell (1975) and Thipayathasana (1975) for the isolation of *unc* mutants. A preparation of the generalized transducing phage P1*kc* was treated with the mutagen hydroxylamine, and used in transduction experiments with strain AN346 (*ilvC*, *unc*$^+$). Transductant strains which could grow in

the absence of isoleucine+valine, were screened for the absence of growth on succinate (Suc^-). Transductants exhibiting the Suc^- phenotype were then tested for low aerobic growth yields in 5mM-glucose-minimal medium.

C. ISOLATION OF STRAINS AN1007 (*unc-436*) AND AN1064 (*unc-441*)

Of the *ilv*$^+$ transductants obtained, 288 colonies were screened for the Suc^- phenotype. Four strains were retained for further observation. One strain, which formed mucoid colonies, had a normal growth yield and was not further investigated. A second strain was found to have a low growth yield (35 Klett units), but grew very slowly on succinate, and was not characterized. Two strains had growth yields characteristic of uncoupled strains, and these were retained for further study. Their growth yields (measured in 5mM-glucose in the absence of Luria broth) were: AN1007 (*unc-436*) 128 Klett units; AN1064 (*unc-441*) 130 Klett units. The presence of the *argH*, *pyrE* and *entA* mutations was checked in both strains.

D. STRAIN AN1065 (*unc-441*): COMPLEMENTATION ANALYSIS

Plasmids containing a known *unc* allele were transferred by conjugation into a recipient strain carrying the uncharacterized *unc-441* allele, using the cross-streaking technique (see Chapter II.E(d)). In order to prevent recombination between the plasmid and the chromosome, a recombination-deficient recipient strain (AN1065) was obtained by mating strain AN1064 (*unc-441*) with the Hfr male strain KL163, which carries the *recA* mutation (see Chapter II.E(c)).

If there is expression of all the genes in the *unc* operon, together with random mixing of the gene products, then strains containing a mutation in a gene on the chromosome different from that affected by the mutant allele on the plasmid, will have a full complement of normal *unc* genes and will grow on succinate. Thus, an uncharacterized *unc* allele can be assigned to a complementation group.

The complementation analysis of strain AN1065 (*unc-441*, *recA*) is shown in Fig. VIII.1. Plasmids carrying the *unc*$^{+}$, *uncB402*, *uncE429*, or *uncC424* alleles complement the *unc-441* mutation. The *unc-441* mutation is not complemented by the plasmid carrying the *uncD409* allele. Complementation with the *uncA401* plasmid is very poor, and colonies are visible only after about 5 days. Control plates containing glucose instead of succinate showed that all plasmids transferred successfully, as judged by the growth of the resulting strains without uracil or arginine, and that strain AN1065 (*unc-441*) tended to revert (results not shown).

E. FURTHER WORK WITH STRAINS CARRYING THE *unc-441* MUTATION

When strain AN1064 (*unc-441*) was grown in batch culture, there were high levels of *unc*$^{+}$ revertants present. The membranes isolated from such preparations contained normal levels of the β-subunit of the Mg-ATPase, and no closely-similar abnormal β-subunit could be distinguished. These membranes had normal NADH-induced atebrin fluorescence quenching, but the rate of ATP-dependent quenching was much slower than in normal membranes, although the levels eventually attained

AN1065

unc-441

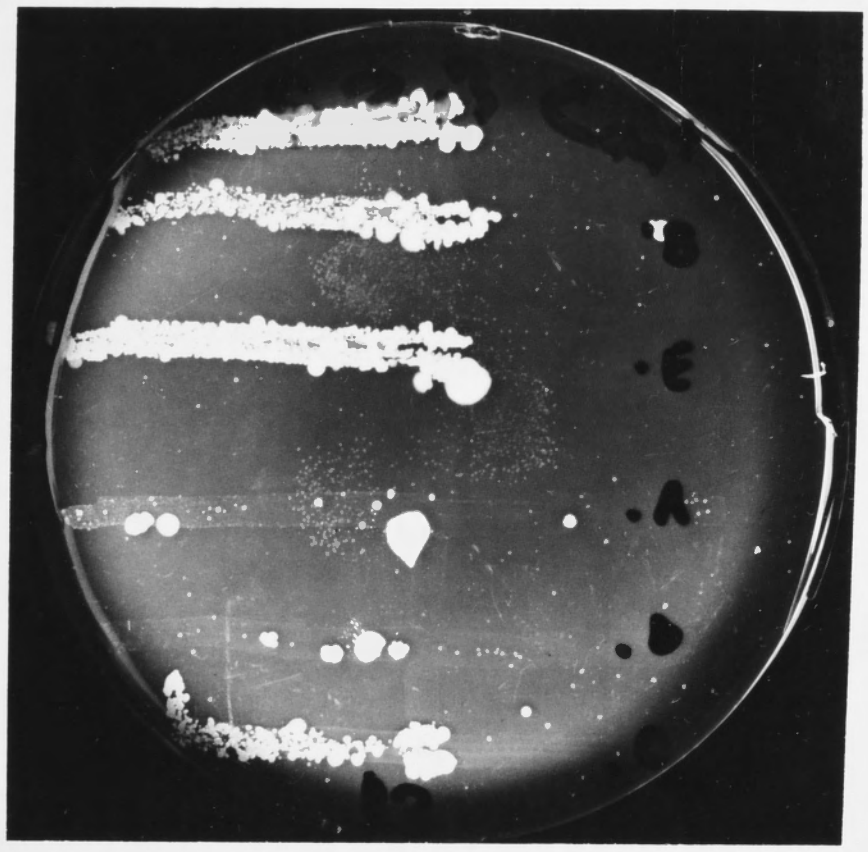


Figure VIII.1 Genetic complementation analysis of strain AN1065 (*unc-441*, *recA*). The recipient strain (AN1065) was spread in the vertical streak, and partial diploid donor strains carrying the following plasmids were streaked across the recipient: (from the top) pAN11 (*unc*$^+$), pAN5 (*uncB402*), pAN13 (*uncE429*), pAN2 (*uncA401*), pAN7 (*uncD409*), and pAN6 (*uncC424*). The plate contained minimal salts medium (Chapter II.B(a)) supplemented with succinate and casamino-acids. The method used is described in Chapter II.E(d).

(40-65%) approached normal values for ATP-dependent quenching. The level of Mg-ATPase activity in these membranes was about 15% of the level in normal membranes, and the Minus-PAB wash from the mutant contained less than 10% of the Mg-ATPase activity found in a normal Minus-PAB wash.

Because strain AN1064 (*unc-441*) proved difficult to grow without excessive reversion, it was not further characterized.

F. STRAIN AN1008 (*unc-436*): COMPLEMENTATION ANALYSIS

The complementation analysis of strain AN1008 (*unc-436*, *recA*) is shown in Fig. VIII.2a. Plasmids carrying the *unc*$^+$, *uncB402*, *uncE429*, or *uncA401* alleles complement the *unc-436* mutation. The complementation is extremely poor with the plasmid carrying the *uncC424* mutation, and the tiny colonies are visible only after about 6 days (Fig.VIII.2a). It has been found that *uncC*$^-$ mutant strains grow very poorly on succinate under some conditions, and thus these results would suggest that strain AN1008 (*unc-436*) was in fact *unc*$^-$. The plasmid carrying the *uncD409* allele did not complement the *unc-436* mutation, which was therefore classified as *uncD*$^-$.

A control plate containing arginine and uracil in addition to the succinate minimal medium, showed that the recipient strain AN1008 (*unc-436*, *recA*, *pyrE*, *argH*) tended to revert to *unc*$^+$ to some extent (Fig. VIII.2b). A further control containing glucose minimal medium showed that all plasmids had transferred successfully (results not shown).

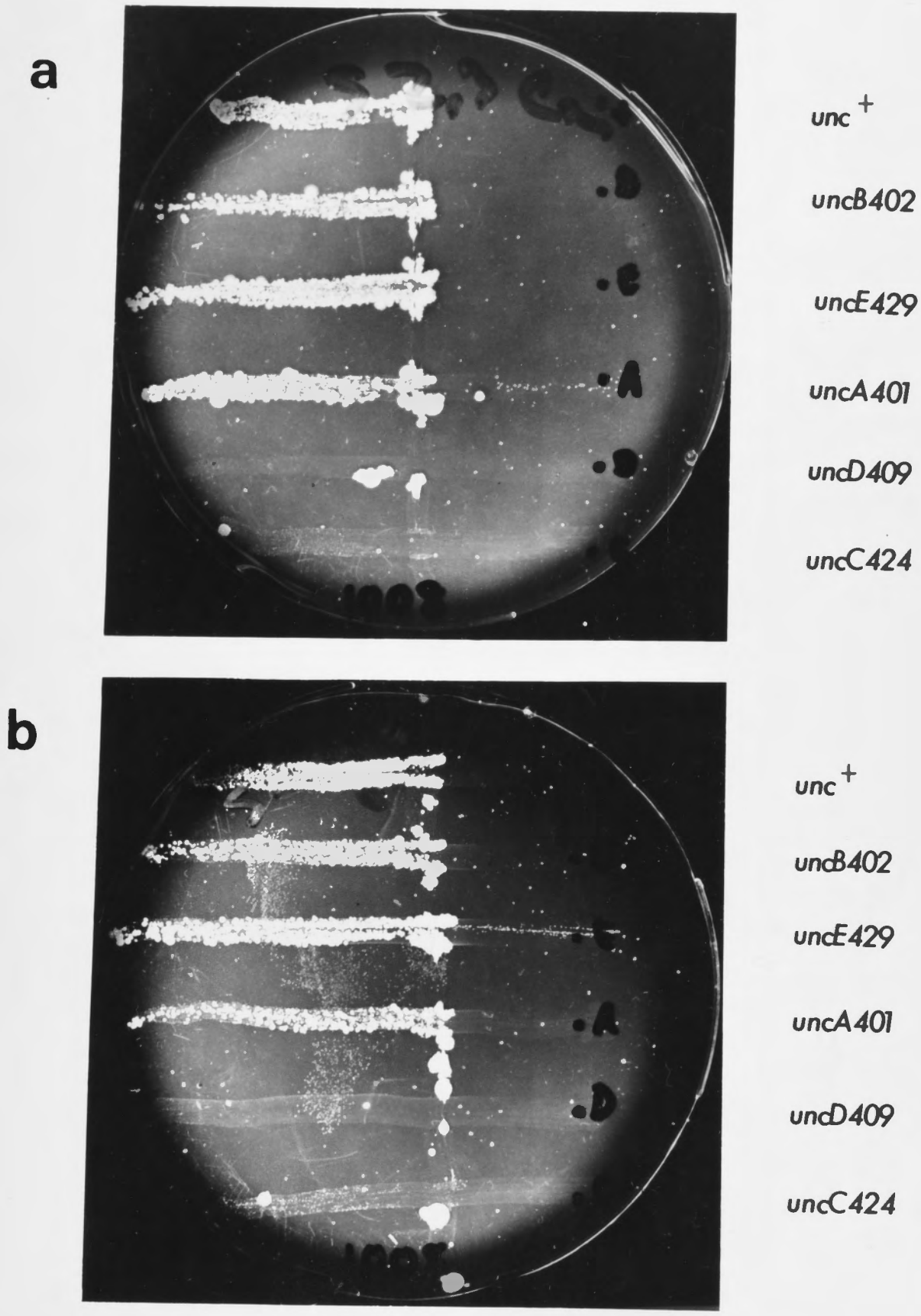


Figure VIII.2 Genetic complementation analysis of strain AN1008 (*unc-436, recA*). The recipient strain (AN1008) was spread in the vertical streak, and partial diploid donor strains carrying the following plasmids were streaked across the recipient: (from the top) pAN11 (*unc*$^+$), pAN5 (*uncB402*), pAN13 (*uncE429*), pAN2 (*uncA401*), pAN7 (*uncD409*) and pAN6 (*uncC424*). Plate (a) contained minimal salts medium supplemented with succinate and casamino-acids, allowing only the growth of strains in which complementation had occurred. Plate (b) contained, additionally, arginine and uracil, which also allowed *unc*$^+$ revertants to grow. The method used is described in Chapter II.E(d).

These results suggested that the *unc-436* mutation was a polar mutation causing the lack of expression of the distal part of the operon, including the *uncD* and *uncC* genes.

G. TEST FOR POSSIBLE SUPPRESSION OF THE *unc-436* MUTATION, BY THE INTRODUCTION OF SUPPRESSOR MUTATIONS INTO STRAIN AN1007 (*unc-436*)

The effects of amber or ochre nonsense mutations, which result in the termination of translation, can often be overcome by suppression of the mutation. A tRNA molecule, charged with its amino-acid, but containing a mutation in the anticodon such that the tRNA now recognises a nonsense codon, can act as a 'suppressor' molecule. Amber suppressors insert their amino-acid in response to an amber nonsense codon (UAG), whereas ochre suppressors recognize ochre codons (UAA), although some ochre suppressors also suppress amber mutations (Garen, 1968).

The bacteriophages $\phi 80Su3^{+}_{am}$ and $\phi 80Su4^{+}_{oc}$, as well as a strain containing a suppressible mutation, W3110 ($trpE9829_{am}$), were kindly provided by Professor C. Yanofsky. Both the above suppressors insert tyrosine (Garen, 1968).

Strains AN1007 (*unc-436*) and W3110 ($trpE9829_{am}$) were each grown to a cell density of about 120 Klett units, and a loopful of each culture was streaked onto a succinate-minimal medium plate (without tryptophan). A spot of a suspension of the phage $\phi 80Su3^{+}_{am}$ was applied to each streak, and in a separate region, a spot of

$\phi 80Su4^{+}_{oc}$ was likewise applied. After 3 days, many colonies were growing in the regions where the control strain ($trpE_{am}$) had been infected by $\phi 80Su3^{+}_{am}$ or $\phi 80Su4^{+}_{oc}$, indicating that the $trpE_{am}$ mutation had been suppressed by both the amber and ochre suppressors. However no colonies had appeared in the infected regions of strain AN1007 (*unc-436*) after 10 days.

H. ENERGY-LINKED ACTIVITIES IN STRAIN AN1007 (*unc-436*)

A comparison of some energy-linked activities of unwashed membranes of strain AN1007 (*unc-436*) and of the 'isogenic' normal strain AN248, is given in Table VIII.1.

(a) Mg-ATPase activity

The Mg-ATPase activity of the unwashed membranes from strain AN1007 (*unc-436*) was negligible compared with that of the unwashed membranes from the normal strain (Table VIII.1). The cytoplasmic fraction from both the mutant and normal strains contained very low amounts of Mg-ATPase activity (results not shown).

(b) Oxidative phosphorylation: P/O ratios

The membranes from strain AN1007 (*unc-436*) had no detectable oxidative phosphorylation, even though the oxidase activities were higher than those of the normal strain (Table VIII.1).

TABLE VIII.1. Comparison of some bioenergetic properties of unwashed membranes from strains AN1007 (*unc-436*) and AN248 (*unc*$^{+}$).

	AN1007 *unc-436*	AN248 *unc*$^{+}$
Mg-ATPase activity (μmol.min^{-1}.mg^{-1})	0.14	1.0 †
P/O Ratio	0.002 *	0.27 §
NADH-induced atebrin fluorescence quenching	80%	78%
ATP-induced atebrin fluorescence quenching	0%	73%
NADH oxidase rate (ng.atoms O.min^{-1}.mg^{-1})	318	255
Lactate oxidase rate (ng.atoms O.min^{-1} mg^{-1})	128	33

* Single determination.

† Data taken from Cox *et al* (1978b)

§ Strain AN822 (*unc*$^{+}$); data from Cox *et al* (1978a).

(c) Atebrin fluorescence quenching

The unwashed membranes of strain AN1007 (*unc-436*) had normal NADH-induced quenching of atebrin fluorescence, but no ATP-induced quenching (Table VIII.1 and Fig. VIII.3a).

I. ATEBRIN FLUORESCENCE QUENCHING IN MEMBRANE FRACTIONS FROM STRAIN AN1007 (*unc-436*)

Membranes from strain AN1007 (*unc-436*) were fractionated by the Minus-PAB washing method outlined in Scheme IV.2b. The EACA-washed membranes retained a low permeability to protons, as judged by the level of NADH-dependent atebrin fluorescence quenching, which was not significantly altered by the addition of DCCD (Fig. VIII.3b). Pre-incubation of the EACA-washed membranes with a normal Mg-ATPase preparation did not reconstitute ATP-induced quenching (Fig.VIII.3c). Lastly, the Minus-PAB wash from strain AN1007 (*unc-436*) was unable to reconstitute NADH-induced quenching in EACA-washed membranes from strain AN248 (unc^+) (Fig.VIII.3d). The PAB-wash from the mutant strain was similarly ineffective in reconstitution (results not shown).

J. POLYPEPTIDE COMPOSITION OF MEMBRANES FROM STRAIN AN1007 (*unc-436*)

It was apparent that the properties of the membranes from strain AN1007 (*unc-436*) resembled those of membranes from strain AN463 (*uncD409*). The polypeptide composition of the membranes from the *unc-436* mutant was therefore investigated.

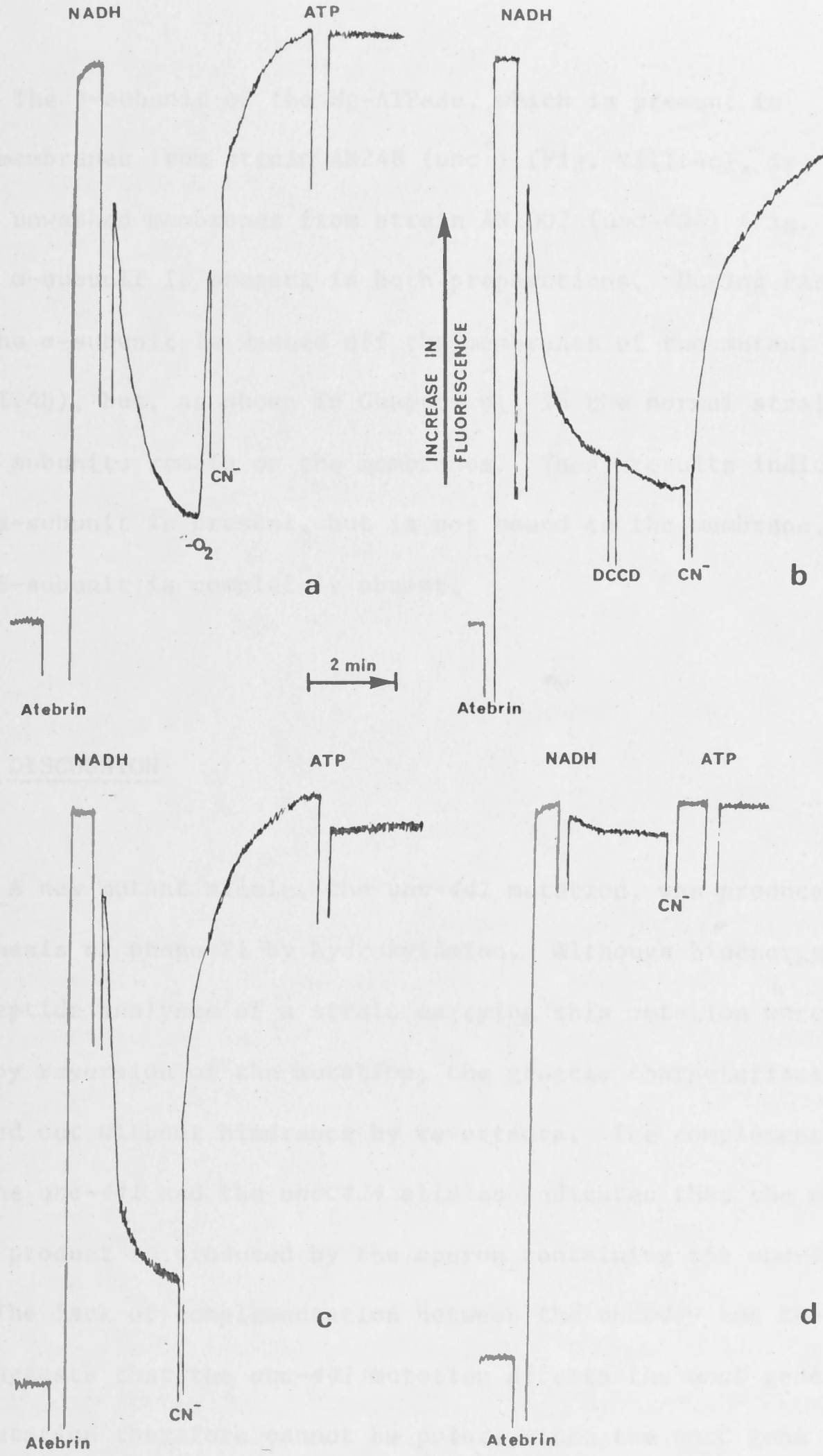


Figure VIII.3. NADH- and ATP-induced atebrin fluorescence quenching in unwashed membranes (a) or EACA-washed membranes (b) from strain AN1007 (unc-436), or (c) AN1007 EACA-washed membranes reconstituted with the Minus-PAB wash from strain AN248 (unc^{+}), or (d) AN248 EACA-washed membranes reconstituted with the Minus-PAB wash from strain AN1007 (unc-436). Details of the reconstitution conditions and the measurement of atebrin fluorescence quenching are given in Chapter II.J. Atebrin was added to the diluted membranes to give a final concentration of 4μM, then NADH (2mM). Where indicated, DCCD (dicyclohexylcarbodiimide) was added at a final concentration of 20μM, NaCN at 2.5mM, ATP at 1mM, and carbonyl cyanide m-chlorophenylhydrazone (CCCP) at 20μM.

The β-subunit of the Mg-ATPase, which is present in unwashed membranes from strain AN248 (*unc*$^{+}$) (Fig. VIII.4c), is absent in unwashed membranes from strain AN1007 (*unc-436*) (Fig. VIII. 4a). The α-subunit is present in both preparations. During PAB-washing the α-subunit is washed off the membranes of the mutant strain (Fig. VIII.4b), but, as shown in Chapter VI, in the normal strain the Mg-ATPase subunits remain on the membranes. These results indicate that the α-subunit is present, but is not bound to the membrane, and that the β-subunit is completely absent.

K. DISCUSSION

A new mutant allele, the *unc-441* mutation, was produced by mutagenesis of phage P1 by hydroxylamine. Although bioenergetic and polypeptide analyses of a strain carrying this mutation were hampered by reversion of the mutation, the genetic characterization was carried out without hindrance by revertants. The complementation between the *unc-441* and the *uncC424* alleles indicates that the normal *uncC* gene product is produced by the operon containing the *unc-441* allele. The lack of complementation between the *uncD409* and the *unc-441* alleles suggests that the *unc-441* mutation affects the *uncD* gene. The *unc-441* mutation therefore cannot be polar, since the *uncC* gene product would then be absent. Therefore the *unc-441* mutation may prove to be a point mutation causing a single amino-acid change in the β-subunit of the Mg-ATPase. This would also be consistent with the known tendency of hydroxylamine to cause a single base-pair alteration (Freese *et al*, 1961). Further investigations into the nature of the lesion caused by the *unc-441* allele will depend on the growth of the strain without contamination by revertants.

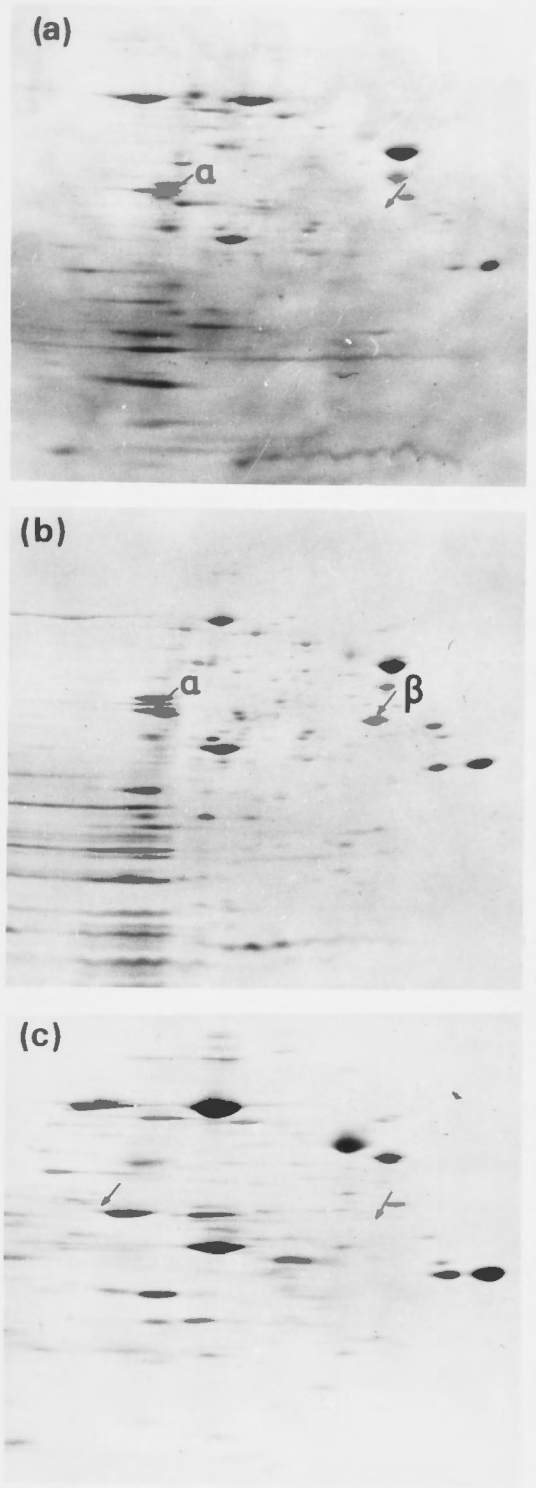


Figure VIII.4 Two-dimensional analytical gel electrophoresis of membranes from strains AN1007 (*unc-436*) and AN248 (unc^+). (a) Unwashed membranes of strain AN1007, 200µg; (b) Unwashed membranes of strain AN248, 196µg; (c) PAB-washed membranes of strain AN1007, 150µg. The α- and β-subunits, or their normal positions, are indicated. The Ampholine composition used in the first dimension gels was 1:1:3 (pH ranges 3.5 to 10: 5 to 7: 4 to 6), and the section of gel shown covers a pH gradient from about 7 to 4.5 (left to right). Details of the methods used are given in Chapter II.L and M.

A second mutant allele, the *unc-436* mutation, was isolated and characterized. Complementation analysis indicated that neither the *uncD* nor the *uncC* genes were expressed in a strain carrying the *unc-436* allele. The *unc-436* mutation was induced by hydroxylamine which is known to react mainly with cytosine, thereby causing a single base pair change (Freese *et al*, 1961). The lack of complementation of the *unc-436* mutation with the *uncD409* or *uncC424* alleles, together with the finding that the *unc* operon is arranged, in order of transcription, *unc B, E, A, D, C* (Gibson *et al*, 1978; J. A. Downie and F. Gibson, unpublished results), suggests that the *unc-436* allele is a nonsense mutation located in the *uncD* gene, or in an unknown gene between the *uncA* and the *uncD* genes. Such a mutation would prevent the translation of both the *uncD* gene and the *uncC* gene.

The observed lack of suppression of the *unc-436* mutation sheds little further light on the nature of the mutation. It is possible that the substitution of tyrosine for the original amino-acid results in a defective gene product, hence the lack of suppression. Alternatively, the *unc-436* mutation may have introduced the codon UGA, a third nonsense codon, which would not be suppressed by the suppressors used (Brenner *et al*, 1967). Lastly, the mutation may have been suppressible, but the introduction of the suppressor may have been lethal for the suppressed strain, although this was not the case for the tryptophan-requiring control strain.

The *unc-436* mutation causes the loss of the β-subunit of the Mg-ATPase in membranes of strain AN1007. The absence of any closely similar polypeptide, such as the abnormal β polypeptides found in membranes of strains AN285 (*uncD405*) or AN463 (*uncD409*), is consistent with the

conclusion that the *unc-436* mutation causes the lack of expression of all, or a considerable portion, of the *uncD* gene, as suggested by the complementation data.

In agreement with this finding, the mutant strain AN1007 (*unc-436*) does not produce a Mg-ATPase aggregate. Strain AN1007 (*unc-436*) is defective in oxidative phosphorylation (but has normal electron transport) and has no Mg-ATPase activity or ATP-dependent energization of the membrane. Neither the Minus-PAB wash nor the PAB wash from the mutant strain are effective in restoring low proton-permeability to stripped membranes from the normal strain. Furthermore, the Minus-PAB treatment of membranes from strain AN1007 (*unc-436*) does not result in an increase in the proton permeability of the membranes.

Normal Mg-ATPase complexes cannot reconstitute ATP-induced energization in the EACA-washed membranes from strain AN1007 (*unc-436*). Further work will be needed to show whether the lack of reconstitution is caused by the lack of a binding site for the Mg-ATPase on the membranes of the mutant, or by the presence of a residual abnormal complex on the membranes which, as in the *uncD409* mutant, cannot be removed by Minus-PAB washing. Such investigations may also help to shed light on the nature of the proton channel which appears to be associated with the membrane-bound Mg-ATPase. If the Mg-ATPase binding site is absent in the *unc-436* mutant, then a polypeptide which is vital for the formation of the proton channel, as well as the polypeptide binding site for the Mg-ATPase, must both be products of gene(s) (perhaps the *uncC* gene) affected by the *unc-436* mutation. If, on the other hand, the *unc-436* mutant has a residual complex bound to the Mg-ATPase binding site and sealing the proton channel, the structure of this complex (and

of the *uncD409* complex), will help to further characterize the polypeptides needed to block such a channel. Partial diploid strains containing combinations of the *unc-436*, *uncD409*, *uncC424* alleles and various Mu-induced *unc* alleles should prove useful in such investigations.

Chapter IX

A COMPARISON OF THE Mg-ATPase OF *Escherichia coli* WITH THE Mg-ATPase (F_1) AND THE F_1-F_0 ATPase COMPLEX FROM THE YEAST *Saccharomyces cerevisiae*

A. INTRODUCTION

In the light of the reported similarities between the membrane Mg-ATPase complexes from many organisms (Haddock and Jones, 1977; see Chapter I), it was of interest to compare the subunit compositions of the enzyme from *E.coli* with that of the Mg-ATPase complex from another organism. Dr. Ivan J. Ryrie kindly provided preparations of the F_1-ATPase and the F_1-F_0 ATPase from the yeast *Saccharomyces cerevisiae* for such a comparison, and the results of such a study are reported in this chapter.

As in other organisms (see Chapter I), the yeast mitochondrial F_1-F_0 ATPase plays a central role in oxidative phosphorylation. The F_1-F_0 ATPase complex has been solubilized from the inner mitochondrial membrane using detergents, and has been purified (Tzagoloff and Meagher, 1971). The Mg-ATPase activity of the F_1-F_0 ATPase complex, like that of the membrane ATPase, is inhibited by oligomycin, which appears to bind to an F_0 sector protein, possibly the DCCD-binding protein (Enns and Criddle, 1977). Recently, the purification method of Tzagoloff and Meagher (1971) has been modified to produce an F_1-F_0 ATPase preparation with higher specific Mg-ATPase activity (Ryrie, 1975b, 1977). The purified F_1-F_0 ATPase, when reconstituted together with phospholipids yielded vesicles which catalysed an electron-transport-independent

ATP-$^{32}P_i$ exchange reaction (Ryrie, 1975a; Ryrie and Blackmore, 1976), and also proton uptake on the addition of Mg^{2+} and ATP (Ryrie and Blackmore, 1976). Furthermore, the F_1-F_0 ATPase vesicles could catalyse ATP synthesis, dependent on energy supplied by an artificial electron transport chain (Ryrie and Blackmore, 1976). In vesicles which also include the light harvesting pigment bacteriorhodopsin, the energy needed for ATP synthesis could be supplied by light (Ryrie and Blackmore, 1976). All three activities, ATP synthesis, ATP-driven proton uptake, and ATP-$^{32}P_i$ exchange, were inhibited by the uncoupler CCCP and by the ATPase inhibitor oligomycin (Ryrie and Blackmore, 1976; Ryrie, 1977). In addition ATPase activity of such vesicles was inhibited by DCCD and stimulated by uncouplers, whereas the solubilized F_1-F_0 ATPase activity was much less sensitive to these compounds (Ryrie, 1975a,b, 1977). Thus the purified F_1-F_0 ATPase complex appears to contain all the polypeptides necessary for ATP synthesis and for ATP-dependent proton pumping, and also any polypeptides responsible for the various effects of inhibitors.

On heating the F_1-F_0 ATPase complex for 2 min at 50°C in the presence of ATP, an F_1-ATPase was released (Ryrie, 1977). This chapter describes the results of a comparison made between the polypeptides present in the purified F_1-ATPase and in the whole F_1-F_0 ATPase, with the polypeptides of the Mg-ATPase from *E.coli*.

B. POLYPEPTIDE COMPOSITION OF THE F_1-ATPase AND THE F_1-F_0 ATPase FROM THE YEAST *Saccharomyces cerevisiae*

(a) Single-dimensional SDS electrophoresis

The polypeptide compositions of the F_1-ATPase and the F_1-F_0

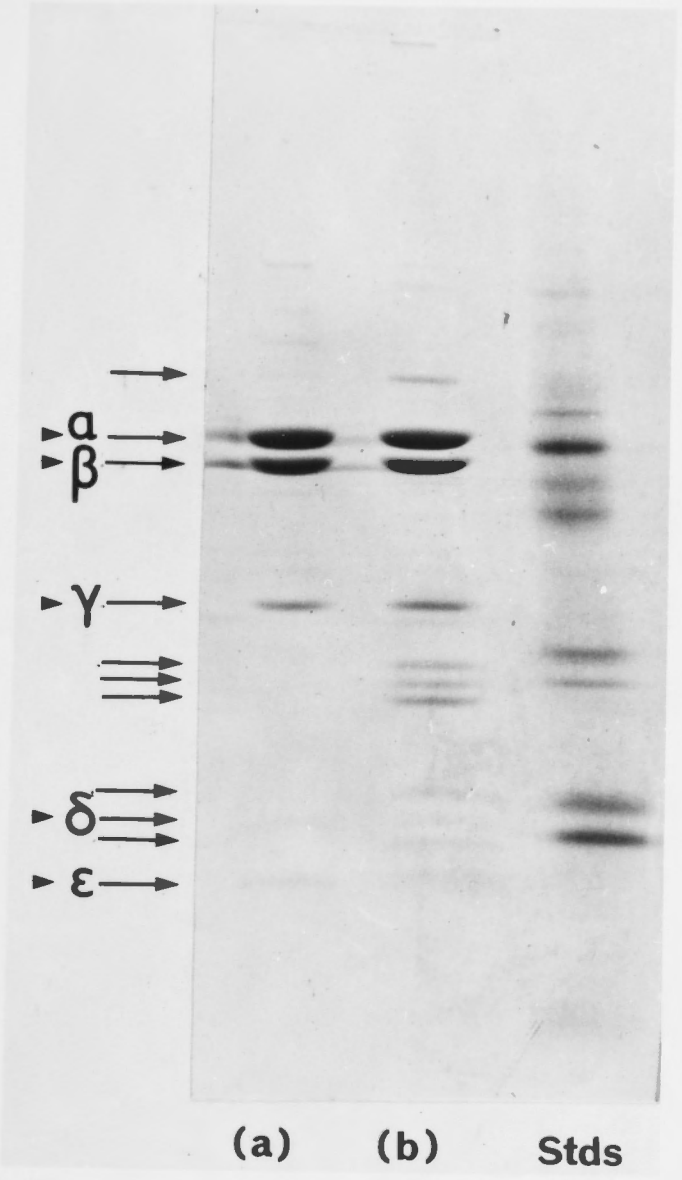


Figure IX.1 SDS electrophoresis of the yeast mitochondrial F_1-ATPase (a; 12μg of protein) and F_1-F_0ATPase (b; 18μg of protein). The positions of the various subunits are indicated. Molecular weight standard proteins (Stds) were: β-galactosidase (120,000), phosphorylase A (93,000), bovine serum albumin (67,000), catalase (60,000), glutamate dehydrogenase (53,000), ovalbumin (43,000), alcohol dehydrogenase (37,000), triosephosphate isomerase (26,500), adenylate kinase (23,000), ribonuclease A (13,500), cytochrome c (12,000). This gel was kindly run by Dr. G. B. Cox.

TABLE IX.1 The apparent molecular weights and isoelectric points of the polypeptides of the purified F_1-F_0 ATPase complex from the yeast *Saccharomyces cerevisiae*.

DESIGNATION OF BAND	APPARENT MOLECULAR WEIGHT	APPARENT ISOELECTRIC POINT	
$F_0(1)$	66,000	5.30	
α	54,000	8.5	(* 6.70, 6.60)
β	48,000	5.60	
("3")	39,000	9 †	
γ	33,000	9	
("5")	30,000	8.8 †	(* 6.9)
$F_0(2)$	25,000	8.5	(* 6.65, 6.75)
$F_0(3)$	22,500	9.4	
$F_0(4)$	21,000	9	
$F_0(5)$	14,000	?5.4	
δ	12,500	5.35	
$F_0(6)$	11,900	-	
ε	10,000	-	
$(F_0(7))$ #	~9,000	-	

* Estimated from the gels of Figs. IX.4 and 5.

† Estimated by comparison of the gels of Fig.IX.2.

\# This band does not stain under the conditions used, but was visible in the gels of Ryrie and Gallagher (in preparation). It may well be the highly non-polar DCCD-binding protein, which reacts poorly with Coomassie blue (Sebald, 1977; Tzagoloff and Meagher, 1971).

ATPase complex from *S. cerevisiae* were studied by single dimensional SDS gel electrophoresis (Fig.IX.1). Samples were dissociated in SDS+ mercaptoethanol in the buffer described in Chapter II.L, at 100°C for 3 min. Standard proteins were also run (Fig.IX.1c), and a molecular weight curve was constructed (cf. Fig.V.5), from which the apparent molecular weights of the components of the F_1-ATPase (Fig.IX.1a) and the F_1-F_0 ATPase (Fig.IX.1b) were calculated. The values obtained are listed in Table IX.1. The F_1-ATPase subunits are denoted α, β, γ, δ, ε, and the F_0 subunits, F_0(1) to (7). Small and variable amounts of band "5" (Table IX.1) were present in all preparations looked at, and band "3" was present in some preparations. The reason for the appearance of these bands will be discussed later in this chapter.

(b) Two-dimensional analytical electrophoresis of the F_1-ATPase

A preparation of the yeast F_1-ATPase was subjected to two-dimensional analytical gel electrophoresis over the acidic pH range (Fig.IX.2). The α-, β- and δ-subunits are indicated; the ε-subunit may have run off the end of the gel. There appears to be some charge modification in this preparation (and in some of the F_1-F_0 ATPase preparations described below) and it is not clear whether the double spot formed by the α-subunit (Fig.IX.2) is an artifact or is actually two closely-similar polypeptides.

(c) Two-dimensional analytical electrophoresis of the F_1-F_0 ATPase

Several preparations of F_1-F_0 ATPase were subjected to two-dimensional analytical gel electrophoresis over the wide pH range (Fig.IX.3). The pattern resulting from two-dimensional electrophoresis of a preparation

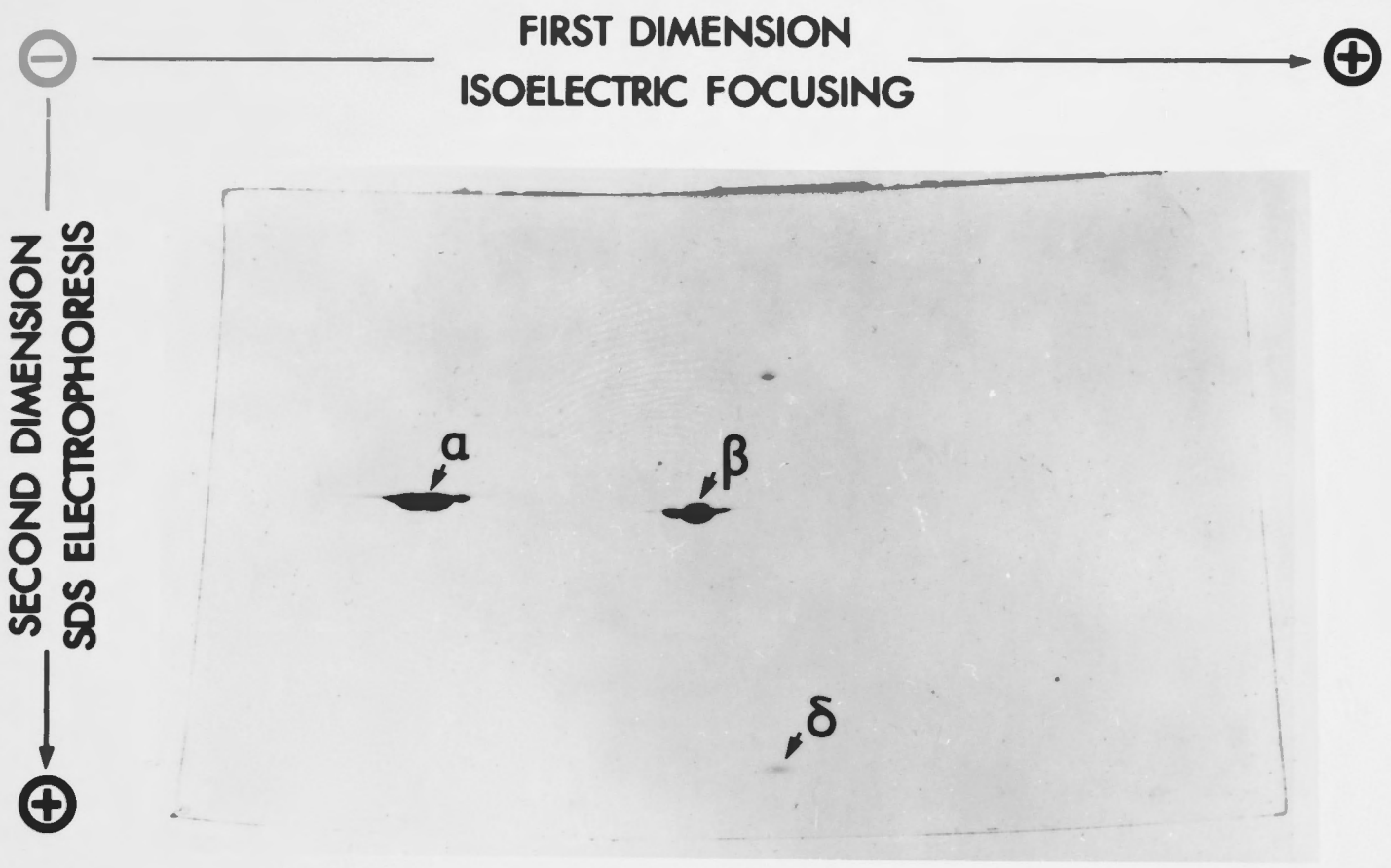


Figure IX.2 Two-dimensional analytical electrophoresis of the yeast mitochondrial F_1-ATPase over the acidic pH range. The sample contained 30µg of protein, The α-, β- and δ-subunits are indicated. The Ampholine composition (i) was used in the first dimension: 1:1:3 (pH ranges 3.5 to 10: 5 to 7: 4 to 6), and other details of the methods are given in Chapter II.M.

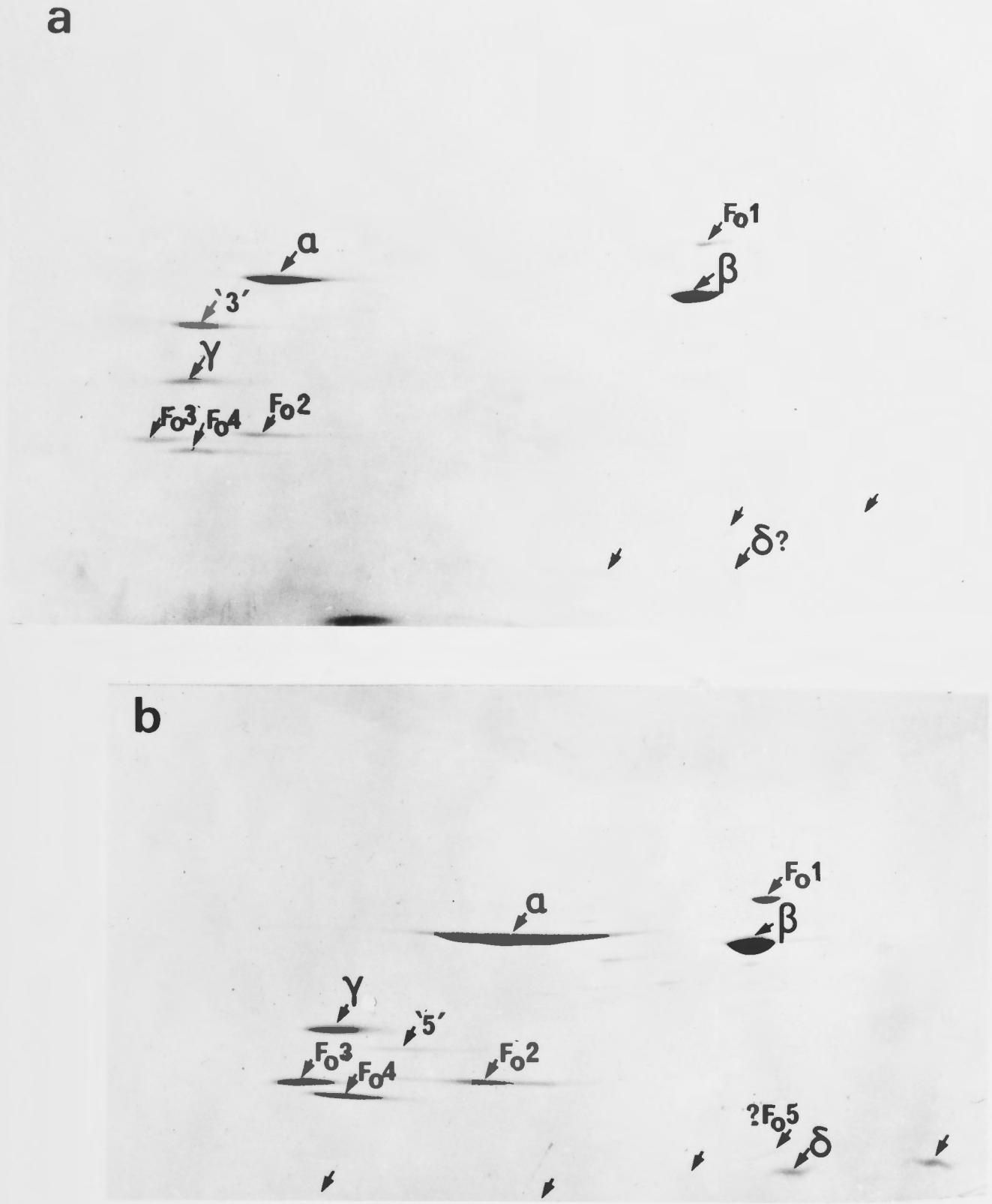


Figure IX.3 Two-dimensional analytical electrophoresis of the yeast mitochondrial F_1-F_0 ATPase over the wide pH range. The samples contained; (a) 30µg of protein, solubilized in SDS + mercaptoethanol at 60°C for 30 min; (b) 60µg of protein, solubilized in SDS + dithiothreitol at 100°C for 3 min, in the presence of protease inhibitors, as described by Ryrie (1977). The subunits which can be identified are indicated, and the positions of detectable low molecular weight polypeptides are arrowed. The Ampholine composition (ii) was used in the first dimension: 1:1:1:2 (pH ranges 3.5 to 5: 5 to 7: 7 to 9: 9 to 11), and other details of the methods are given in Chapter II.M. The anode buffer for gel (a) was 0.1M-H_3PO_4, and for gel (b) was 0.01M-H_3PO_4.

solubilized at 60°C for 30 min in the absence of protease inhibitors, and therefore containing band "3" (see below) is shown in Fig.IX.3a. The positions of the F_1-ATPase subunits α, β, γ, and δ are indicated, as well as the positions of band "3", and the F_0 subunits $F_0(1)$, $F_0(2)$, $F_0(3)$ and $F_0(4)$. The positions of the low-molecular weight polypeptides (including the δ-subunit) were disturbed by the presence of detergent micelles in this area of the gel, making identification difficult. The positions of some detectable polypeptides are indicated.

It has recently been observed that the presence of protease inhibitors throughout the preparation (Ryrie, 1977) and subsequent SDS dissociation (I. J. Ryrie and A. Gallagher, in preparation) of the F_1-F_0 ATPase resulted in a preparation lacking band "3". In agreement with these results, the two-dimensional gel pattern of F_1-F_0 ATPase prepared in this way lacks band "3" (Fig.IX.3b; cf. Fig.IX.1b). The other subunits are also indicated, and traces of band "5", which was barely detectable in the preparation of Fig.IX.3a can also be seen. The appearance of bands "3" and "5" has been investigated by Ryrie (1977), and details of these findings will be discussed later in the chapter. The apparent isoelectric points of several of the subunits were determined from an isoelectric focusing gel similar to that used in the experiment shown in Fig.IX.3b, and these values are listed in TableIX.1. The pattern obtained by electrophoresis over the acidic pH range of a similar F_1-F_0 ATPase preparation (lacking band "3") is shown in Fig.IX.4. The α-, β- and δ-subunits and the $F_0(2)$ subunit are present, together with traces of band "5" and a number of unidentified low molecular weight bands. The double spot formed by the α-subunit is again present.

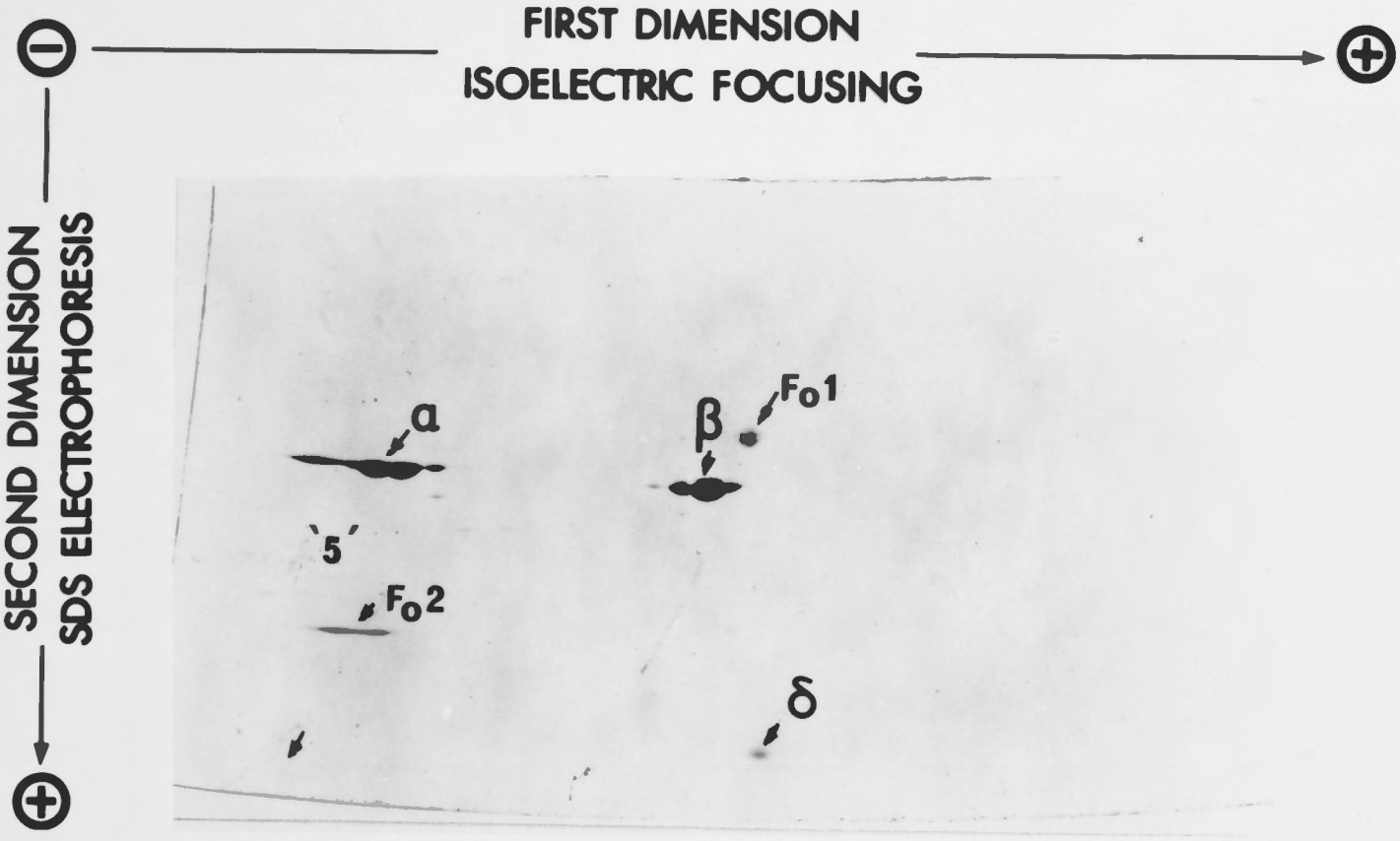


Figure IX.4 Two-dimensional analytical gel electrophoresis of the yeast mitochondrial F_1-F_0 ATPase over the acidic pH range. The sample contained 60µg of protein, solubilized as described for Fig. IX.3b. The subunits identified are indicated, as well as the positions of several low molecular weight polypeptides. The Ampholine composition (i) was used in the first dimension: 1:1:3 (pH ranges 3.5 to 10: 5 to 7: 4 to 6), and other details of the method are given in Chapter II.M.

C. COMPARISON OF THE POLYPEPTIDE PATTERNS OBTAINED AFTER TWO-DIMENSIONAL ELECTROPHORESIS OF THE Mg-ATPase FROM *E.coli* TOGETHER WITH THE F_1-ATPase OR THE F_1-F_0 ATPase FROM *Saccharomyces cerevisiae*

In the light of the obvious similarities between the *E.coli* Mg-ATPase subunits and those of the yeast mitochondrial F_1-ATPase, the yeast F_1 or F_1-F_0 ATPase was run, together with a small amount of the *E.coli* Mg-ATPase, on a two-dimensional gel in an attempt to gain further information on the similarities and differences between the preparations. Both wide pH range (Fig.IX.5a) and narrow pH range (Fig.IX.5b) gels were used. The molecular weights of the α- and β-subunits of the Mg-ATPases from the two organisms are identical within the limits of resolution of the gels, thus confirming the values previously assigned (Table IX.1) of 54,000 for the α-subunit and 48,000 for the β-subunit (see also Fig.V.5). The α- and β-subunits of the *E.coli* Mg-ATPase have more acidic apparent isoelectric points (6.25 and 5.35 respectively; see Fig.V.7) than their counterparts from yeast (Fig.IX.5; Table IX.1). The γ-subunit from the Mg-ATPase of *E.coli* can also be detected on the wide pH range gel (Fig.IX.5a). Its apparent isoelectric point is slightly more acidic (pI 8.9, see Chapter V) than that of the yeast γ-subunit, which was found to have a pI of about 9 (Table IX.1). The molecular weight of the yeast ATPase γ-subunit is slightly higher (33,000) than that of the *E.coli* counterpart (30,000; see Fig.V.5), and this difference is seen in the gel Fig.IX.5a. There is insufficient *E.coli* Mg-ATPase on the gels of Fig.IX.5 to detect the δ-subunit, but the δ-subunit from the yeast F_1-F_0 ATPase can be detected in both the acidic (Fig.IX.5b) and wide (Fig.IX.5a) pH range gels. The isoelectric point of the yeast ATPase δ-subunit was similar to that of the *E.coli* Mg-ATPase β-subunit, in agreement with the

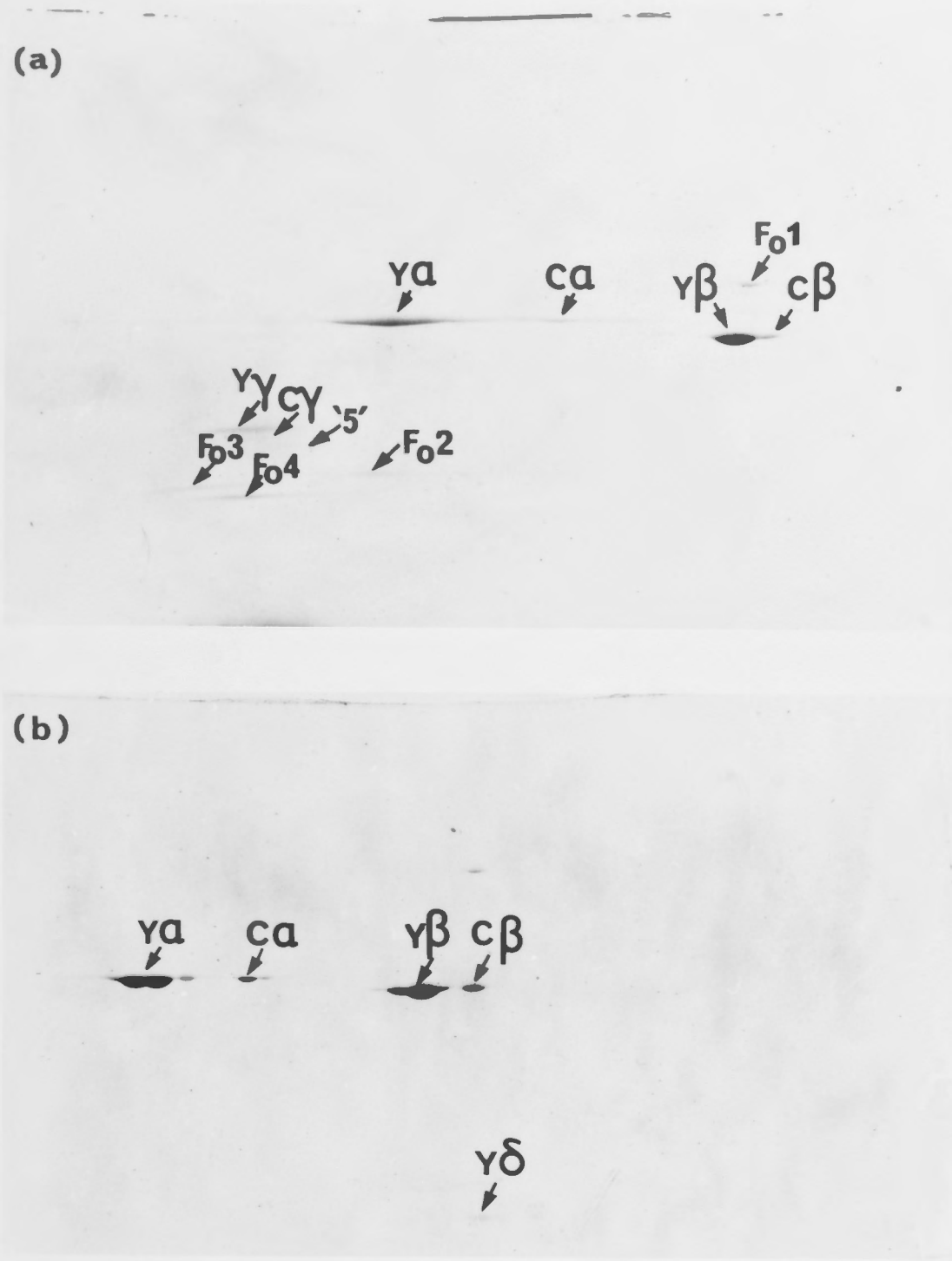


Figure IX.5 Two-dimensional analytical gel electrophoresis of (a) a mixture of the yeast mitochondrial F_1-F_0 ATPase and the *E.coli* Mg-ATPase (F_1) over the wide pH range; and (b) a mixture of the yeast mitochondrial F_1-ATPase and the *E.coli* Mg-ATPase (F_1) over the acidic pH range. The amount of *E.coli* Mg-ATPase protein in both gels was about 2µg; the amount of the yeast preparations were: gel (a) 30µg of protein; gel (b) 20µg of protein. The Ampholine compositions used in first dimension were: (a) 1:1:1:2 (pH ranges 3.5 to 5: 5 to 7: 7 to 9: 9 to 11); (b) 1:1:3 (pH ranges 3.5 to 10: 5 to 7: 4 to 6). The yeast proteins were solubilized as outlined in Fig. IX.3b. Other details of the methods are given in Chapter II.M. The yeast polypeptides are denoted "Yα" etc, and the *E.coli* polypeptides "Cα" etc.

pI values of 5.35 which had been assigned independently to both polypeptides (Table IX.1 and Fig.V.7). The δ-subunit of the *E.coli* Mg-ATPase also had an apparent pI of 5.3 to 5.4 (Fig.V.7), although its molecular weight (20,000; Fig.V.5) was substantially higher than that of the yeast ATPase δ-subunit (12,500; Table IX.1). The ε-subunit could not be positively identified in gels of the F_1-F_0 ATPase polypeptides. In such gels, however, two spots were seen at positions corresponding to molecular weights of about 10,000 and isoelectric points of about 6.1 and 6.4 (Fig.IX.3, 4 and 5). The ε-subunit of the *E.coli* Mg-ATPase was shown in Chapter V to have a molecular weight of 12,000 and an apparent pI of 5.95.

D. ELECTROPHORESIS OF THE YEAST F_1-F_0 ATPase UNDER NON-DISSOCIATING CONDITIONS: PRELIMINARY EXPERIMENTS

The results of preliminary experiments in which the yeast F_1-F_0 ATPase complex was subjected to electrophoresis under non-dissociating conditions, should be noted. In a large-pore acrylamide gradient gel (T = 5.25 to 6.75%, C = 5.7%), a band of Mg-ATPase activity was seen at an apparent molecular weight of 450,000, estimated by comparison with the chloroform-solubilized Mg-ATPase complexes from *E.coli* run on the same gel (cf.Fig.V.3). A molecular weight for the F_1-F_0 complex of 520,000 (including lipid) was estimated from the sedimentation coefficient by Tzagoloff and Meagher (1971). Kagawa *et al*, (1976) purified a $TF_0.F_1$ ATPase from the thermophilic bacterium PS3, and determined the molecular weights and stoichiometry of its subunits. The resulting molecular weight total comes to 458,000. Hare (1975) purified a complex from *E.coli* of similar size as judged by its sedimentation

velocity through a sucrose gradient.

When the yeast F_1-F_0 ATPase preparation was run on a smaller-pore gel (T = 6 to 7.5%, C = 5.7%), a band was seen at the same apparent molecular weight ('310,000') as the higher-mobility chloroform-solubilized Mg-ATPase from *E.coli*. A second band was seen at a mobility corresponding to a molecular weight of 270,000. It is possible that the heat generated in the latter gel was sufficient to release the F_1-ATPase from the complex, since one method of preparation of F_1-ATPase is by the heat-induced splitting of the F_1-F_0 complex (Ryrie, 1977). Tzagoloff and Meagher (1971) estimated the molecular weight of their F_1-ATPase complex at 340,000, from the sedimentation coefficient.

E. DISCUSSION

The comparison made between the subunits of the Mg-ATPase from *E.coli* and the analogous subunits of the yeast mitochondrial F_1-ATPase, extends the observations made previously, that Mg-ATPase complexes from diverse organisms are very similar in structure and subunit composition (Senior, 1973; Pedersen,1975; Haddock and Jones, 1977; Sebald, 1977). The striking resemblances between the two-dimensional gel patterns of the *Saccharomyces cerevisiae* F_1-ATPase and the *E.coli* Mg-ATPase can be attributed to similarities in the structure of the native enzymes, since for each subunit in one complex, a counterpart can be found in the other with a comparable isoelectric point and molecular weight, although such a relationship has not yet been established for the ε-subunit.

Knowles and Penefsky (1972a,b) reported that the δ-subunit of the beef heart mitochondrial Mg-ATPase had a molecular weight of approximately 17,000, and an apparent isoelectric point of 5.2, determined in the presence of urea. The isoelectric point is thus similar to those obtained in the present work for the δ-subunits from both the yeast mitochondrial F_1-ATPase (pI 5.35) and the Mg-ATPase from *E.coli* (pI 5.3 to 5.4), whilst the molecular weight of the beef heart δ is intermediate between the molecular weights of the yeast and *E.coli* δ polypeptides.

The anomalous behaviour of the α-subunit during isoelectric focusing over acidic or wide pH ranges (cf.Figs.IX.3a, 3b and 4) was also noted with the α-subunit from the *E.coli* enzyme (see Chapter V.D(c) and Fig.V.11). Like the *E.coli* polypeptide, the yeast F_1-F_0 ATPase α-subunit focuses either in the mildly acidic region, or at about pI 8.5, or streaks out between these values, depending on the conditions used for electrophoresis and for solubilization of the sample. Unlike the *E.coli* polypeptide however, the yeast F_1-F_0 ATPase α-subunit appeared to focus in two bands in the acidic region. Further work will be necessary to determine whether or not this is an artifact. Both species of α-subunit, in approximately equal proportions, were also seen in gels of the F_1 preparation.

Sebald (1977) found that the molecular weight of the α-subunit from the yeast mitochondrial ATPase (*S. cerevisiae*) had the same mobility in an SDS slab gel as the α-subunits of mitochondrial Mg-ATPases from beef heart, rat heart and the mould *Neurospora crassa*. A similar relationship applied for the β-subunit. Hence, the molecular weights of the α- and β-subunits of the *E.coli* Mg-ATPase are indistinguishable from

those of mitochondrial ATPases from four organisms.

Thus, during evolution, the properties of the Mg-ATPase subunits would appear to have been remarkably tightly-conserved, suggesting that the functioning of this enzyme is closely-dependent on these properties. Perhaps the correct assembly of the whole complex as a part of the membrane requires a finely balanced set of charge interactions and molecular volumes. If this balance were to be disturbed (for instance by mutation), then correct assembly might not take place and activity would then be lost. It is also likely that proton pumping, a function which appears to be intimately involved with ATP synthesis and hydrolysis by the F_1-F_0 complex (see Chapter I), also depends on the correct spatial organisation of the charges on some of the subunit polypeptides.

The numbering systems previously applied to the bands of the F_1-F_0 ATPase complex from yeast mitochondria (Tzagoloff and Meagher, 1971; Ryrie, 1977), have recently been shown to be inadequate for a number of reasons (I. J. Ryrie and A. Gallagher, in preparation; Ryrie, 1977). Firstly, band "3" of Tzagoloff and Meagher (1971) does not appear when the complex is prepared thoughout in the presence of 5mM-PAB, 5mM-EACA, and a third protease inhibitor, phenylmethylsulphonyl fluoride (0.5mM) (Ryrie, 1977). All of the F_1-F_0 ATPase complexes used in the present work were prepared in this way. However, a preparation solubilized at 60^{o}C for 30 min in the absence of protease inhibitors (Fig.IX.3a) contained band "3". This puzzling observation was explained by the identification, by I. J. Ryrie and A. Gallagher (in preparation), of band "3" as a breakdown product of the α-subunit, caused by the SDS-induced activation of a latent protease contaminant which is present in

yeast F_1-F_0 preparations. Band "3" was also obtained if protease inhibitors were omitted during preparation of the F_1-F_0 ATPase (Ryrie, 1977).

The origin of band "5" is unknown, and only traces of it are normally seen in F_1-F_0 ATPase prepared and treated with SDS in the presence of protease inhibitors (Ryrie, 1977). A good candidate would be the adenine nucleotide translocase, which has a molecular weight of about 30,000 in *Neurospora crassa* (Sebald, 1977), in rat heart (Lauquin *et al*, 1976) and beef heart (Riccio *et al*, 1975) mitochondria. In rat liver mitochondria, the adenine nucleotide translocase *in situ* is closely associated with the ATPase (Vignais *et al*, 1975) but in beef heart mitochondria, the translocase can be separated from the F_1-F_0 ATPase (Serrano *et al*, 1976).

The occurrence of proteases in yeast, and in particular of an SDS-activated protease, has been reviewed by Pringle (1975). A review by Holzer *et al* (1975) lists the information available on the proteases of many micro-organisms, including *E.coli* and *Saccharomyces cerevisiae*.

Band "3" was considered by other workers to be the third of five F_1 subunits (Tzagoloff and Meagher, 1971), and this view has persisted (Senior, 1973; Pedersen, 1975; Haddock and Jones, 1977). Takeshige *et al* (1976) prepared a five subunit enzyme without band "3", and a six subunit complex containing this band. The present work favours the views of Goffeau *et al* (1973), Takeshige *et al* (1976) and I. J. Ryrie and A. Gallagher (in preparation) that nomenclature for the F_1-ATPase should disregard band "3", that band "4" of Tzagoloff and Meagher (1971)

should now be designated "γ", and that band "8" of the F_1-ATPase of Tzagoloff and Meagher (1971) consists of the δ- and ε-subunits. It must be stressed that the new numbering system used here for the F_0 polypeptides does not correspond to previous numbering systems for the F_1-F_0 polypeptides (Tzagoloff and Meagher, 1971; Ryrie, 1977). It should also be emphasized that, although all the F_0 polypeptides are present, one or more of the polypeptides designated F_0(1) to (7) may not be a necessary component.

Chapter X

GENERAL DISCUSSION

The main findings of the work presented in this thesis were discussed in the concluding sections of each chapter, and were summarized in the Abstract (pg.viii). This chapter is intended only to present some speculative ideas based on the findings presented in the preceding chapters, together with the work of others.

(a) The *unc* operon and subunit stoichiometry

The detection of abnormal Mg-ATPase β-subunits in the *uncD409* and *uncD405* mutants (Chapters VI and VII), and the absence of the β polypeptide in the *unc-436* (*uncD⁻C⁻*) mutant, provide conclusive evidence that the *uncD* gene is the structural gene for the β-subunit. This is the first gene-polypeptide relationship established for an *unc* gene, although the identities of polypeptides altered or missing are known for a number of strains with mutations mapping in the *unc* region (e.g. strains BG-31, *etc-15*, RF-7, N_{I44}, NR-70; Tables I.1 and 2).

No other *unc* gene-polypeptide relationship has yet been established. However the identity of the gene coding for the α-subunit has been narrowed down to a section of the operon (ordered *uncBEADC*; see Chapters I and VIII) distal to the *uncE* gene and before the *uncD* gene. The evidence for this contention comes from two observations. Firstly, the polar mutant *unc-436* (*unc D⁻C⁻*) produces a normal α-subunit (see Chapter VIII) which, although attached to the membrane very loosely or not at all, is nevertheless not degraded. Secondly,

in preliminary investigations (D. Fayle, results not presented) into the polypeptide composition of unwashed membranes from a strain carrying the *unc-418*:: Mu mutation (see Table I.3), which is *uncB*$^{+}$*E*$^{+}$*A*$^{-}$*D*$^{-}$*C*$^{-}$, it was found that the α-subunit was completely absent. The β-subunit was also absent, as predicted from the *uncD*$^{-}$ genotype. The only gene so far known to be located between the *uncE* and *uncD* genes is *uncA*, though there may well be others. The α-subunit of the Mg-ATPase of the *uncA401* mutant was indistinguishable from the normal α-subunit (see Chapter VII). It would clearly be of advantage to isolate further point mutants affected in the *uncA* gene.

The identification of the gene coding for the β-subunit, together with the localization of the region containing the gene coding for the α-subunit, has some implications in terms of the biosynthesis of the Mg-ATPase. It was already noted that the *unc-415*::Mu mutant strain possessed Mg-ATPase activity (Table I.3). This strain is *uncB*$^{+}$*E*$^{+}$*A*$^{+}$*D*$^{+}$*C*$^{-}$, and it is therefore clear that all of the genes necessary for the synthesis of the ATP-hydrolytic activity are transcribed before the *uncC* gene. As the β-subunit is specified by the last known gene in this region of the operon, *uncD*, it is likely that at least the *uncA*, *uncE* and *uncB* gene products, and the α-subunit, are produced in numbers equal to (or greater than) the number of β-subunits produced. If the structural genes for the minor subunits of the Mg-ATPase are also in this region, it would suggest that the stoichiometry of the F_1 subunits (α:β:γ:δ:ε) is 2:2:2:2:2 rather than 3:3:1:1:1 (see Chapter I).

Vogel and Steinhart (1976) suggested that the stoichiometry of the subunits was either 2:2:2:1:2 or 2:2:2:2:2. The possibility that there are unstoichiometric amounts of δ-subunit was also indicated in Chapter V, and in the work of others, summarized in Chapter V.F. Two

possible reasons for such observations come to mind. One is that the δ-subunit may be easily lost during isolation of the enzyme (see Chapter V). The other is that there may be two such populations of Mg-ATPase *in vivo*. This explanation would require that the structural gene for the δ-subunit was either outside the operon, or was distal to the *uncD* gene, so that the α- and β-subunits could be produced in greater numbers than the δ-subunit.

The observation in Chapter V on the subunit structures of two separable species of chloroform-solubilized Mg-ATPase indicate that these species could be $(2\alpha, 2\beta, 2\gamma, 1\varepsilon)$ and $(2\alpha, 2\beta, 2\gamma, 0\varepsilon)$, although this is by no means proven. By comparison, the low-ionic strength-solubilized Mg-ATPase would be $(2\alpha, 2\beta, 2\gamma, 1\text{-}2\delta, 2\varepsilon)$. This interpretation of the results is based on a 2:2:2:1-2:2 stoichiometry; the results are a little harder to interpret if there is only one copy of the ε-subunit, as required by a 3:3:1:1:1 stoichiometry.

A further comment on the stoichiometry of the Mg-ATPase components relates to the DCCD-binding protein. As noted in Chapter I, several workers have suggested that the DCCD-binding protein of *E.coli* could exist as a trimer *in situ* (Fillingame, 1976; Altendorf, 1977). The protein has a known tendency to polymerize, even in SDS (Nieuwenhuis and Bakkenist, 1977). In yeast mitochondria, the DCCD-binding protein appears on SDS gels either as a hexamer of 45,000 daltons, or as a monomer of 7,500 daltons, depending on the treatments applied during its preparation (Tzagoloff and Akai, 1972; Sierra and Tzagoloff, 1973; Tzagoloff *et al*, 1973). An analogous hexamer of the *E.coli* DCCD-binding protein (M.W. 9000) would have a molecular weight of around 54,000. Interestingly, the protein missing in the *unc* ATPase$^+$ strain BG-31 (Simoni and Shandell, 1975) is of 54,000 daltons, and is not

the α- or β-subunit of the Mg-ATPase. The amber mutation does not affect the transcription of the *uncD* gene; presumably a gene or genes distal to *uncD* are affected. A novel protein of about half the molecular weight was produced. The Mg-ATPase binding site was concomitantly affected, rendering the Mg-ATPase insensitive to DCCD, and the membranes did not become leaky to protons after low-ionic strength treatment (Simoni and Shandell, 1975; Table I.2).

If such a DCCD-binding protein hexamer exists, and it is in a 1:1 ratio with the F_1-sector of the Mg-ATPase, then the gene coding for the DCCD-binding protein might be out of the operon, or perhaps at the beginning of it. Alternatively, the hexamer could be made up of three pairs (or two triplets) of hydrophobic polypeptides of very similar molecular weight, one pair (or triplet) of which is the DCCD-binding protein.

Other work leading to the proposal of stoichiometries for the subunits is summarized in Senior (1978). Verschoor *et al* (1977) recently concluded that there were two β-subunits per mitochondrial Mg-ATPase complex, on the basis of aurovertin-binding studies.

(b) <u>The size of the F_1-portion of the Mg-ATPase</u>

If a subunit stoichiometry of 2:2:2:2:2 is assumed for the complete F_1-portion of the Mg-ATPase, and assuming the accuracy of the subunit molecular weight determinations, a molecular weight of 328,000 for the complex can be calculated. Muller *et al* (1977) have calculated the molecular weight of the beef heart mitochondrial enzyme by several methods, and concluded that 8% of the weight of the complex was material that was neither protein, nucleotide, or Mg^{2+}. If a

similar situation is assumed for the *E.coli* enzyme, the total molecular weight comes out to 354,000. The corresponding values for a 3:3:1:1:1 stoichiometry are 368,000 and 397,000. The value estimated by pore-gradient electrophoresis (Chapter V) was 360,000. More information is therefore needed before any inference as to stoichiometry or the true size of the complex can be made.

(c) The roles of the F_1-subunits

The data presented in this thesis do not allow any conclusion to be drawn as to the location of a catalytic site in the β-subunit (see Chapter I; Kozlov and Skulachev, 1977). In the two *uncD* point mutants investigated in detail (*uncD409* and *uncD405*), the mutation resulted in the lack of assembly of a Mg-ATPase aggregate. A similar situation applies with regard to the polar $uncD^-C^-$ mutants, where there is no β-subunit. In such a situation, the lack of catalytic activity is hardly surprising. The *uncA401* mutant produces a structurally-normal aggregate, but the polypeptide affected by the mutation has not yet been identified. In this regard, strain AN1064 (*unc-441;* $uncD^-C^+$) described in Chapter VIII could prove useful. From the experiments done so far, the strain appears to produce an otherwise normal aggregate, lacking Mg-ATPase activity (Chapter VIII.E).

The model for reversible ATPase activity proposed by Kozlov and Skulachev (1977) depicts the α-subunit as having the non-catalytic binding site for ADP+P_i or ATP, whilst the β-subunit contains a catalytic site which is not directly accessible to nucleotides. At the same time as this model was published, Yoshida *et al* (1977a), working with the thermostable TF_1-F_0, published their finding that the reconstituted (β+γ) and (β+γ+δ+ε) complexes had Mg-ATPase activity. An additional

observation was that reconstituted vesicles containing TF_0+($\beta+\gamma+\delta+\varepsilon$) carried out only a slow ATP $\rightleftharpoons$ $^{32}P_i$ exchange (Yoshida *et al*, 1977b), whilst the TF_0+($\alpha+\beta+\gamma+\delta+\varepsilon$) catalysed the ATP $\rightleftharpoons$ $^{32}P_i$ exchange at much faster rates. In membranes from *E.coli*, the addition of antiserum to the α-subunit affected the ATP $\rightleftharpoons$ $^{32}P_i$ exchange and Mg-ATPase activity more effectively than did antiserum to the β-subunit (Kanner *et al*, 1975). These results are consistent with the suggestion of Kozlov and Skulachev (1977) that the α-subunit contains the initial binding site for P_i+ADP, whilst the β-subunit contains a catalytic site.

The tentative conclusion, drawn in Chapter V, that the chloroform-solubilized Mg-ATPases lack one or more copies of the ε-subunit, suggests a method for investigating whether in fact the ε-subunit does suppress Mg-ATPase activity. Interestingly, Yoshida *et al* (1975) found that chloroform treatment of purified TF_1-ATPase activated the enzyme.

Finally, the lack of any δ-subunit, together with the suggested loss of one or more ε-subunits, in the chloroform-solubilized Mg-ATPases, can be correlated with the work of Yoshida *et al* (1977b), who concluded that the δ- and ε- subunits were necessary for the binding of the major subunits to the TF_0-sector. The chloroform-solubilized Mg-ATPases were unable to reconstitute any activity in stripped membranes, suggesting that they cannot bind to the F_0-sector.

(d) The site of action of the protease

It can be presumed that the action of *p*-aminobenzamidine in preventing the solubilization of the Mg-ATPase is related to its potent effect on trypsin-like proteases. The question of the site of action of

the presumptive protease then arises.

One possibility is that the δ-subunit is a product of such a protease. What, then was the native polypeptide from which the δ-subunit arises? One possibility is that there is a very slightly larger polypeptide, of pI about 5.3, which is seen in membrane preparations and in highly-purified Mg-ATPase (see e.g. Fig. V.7a). One could envisage a site susceptible to proteolytic attack near the end of this polypeptide, which when cleaved, produces the δ-subunit. As there are apparently Mg-ATPase complexes containing two δ-subunits, and some of this "native" polypeptide is found in the purified Mg-ATPase, it may be necessary to cleave only one δ-subunit to solubilize the Mg-ATPase. The alternate possibility of charge modification should be noted however.

A second possibility is suggested by the work of Kozlov and Mikelsaar (1974), who isolated a mitochondrial Mg-ATPase apparently containing only the α, β and ε subunits. These workers suggested that the (γ+δ) was, *in vivo*, a subunit of 50-60,000 daltons, resembling the α- and β-subunits. The observation that the chloroform-solubilized Mg-ATPase contains the γ-subunit but not the δ-subunit, does not eliminate this possibility, since the δ cleavage product could conceivably be extracted into the insoluble or chloroform fractions. This hypothetical site of cleavage must also be conserved during evolution since the γ- and δ-subunits of the yeast mitochondrial Mg-ATPase bear a close resemblance to the *E.coli* polypeptides (see Chapter IX).

Lastly, it should be stressed that the site of action of the presumed protease may well be elsewhere in the Mg-ATPase complex, but

apparently not in the α- or β-subunits, since these are present both in the PAB-washed membranes and in the purified Mg-ATPase.

(e) The behaviour of the altered β-subunits in *uncD* mutants

The altered β-polypeptides of the *uncD409* and *uncD405* mutants were not removed from the membrane during the low-ionic strength washing procedure (without PAB). (In this discussion, 'altered β-subunit' can be taken to include the minor subunits (γ, δ or ε) if these are also present). One inference which may be drawn is that the normal proteolytic cleavage site is absent or altered in these strains, even though the mutation affects the β-subunit.

A more remarkable phenomenon is the effect of these abnormal polypeptides on proton movement through the F_0-sector. In both mutants, the F_0-sector is presumed to be intact. As discussed in Chapter VII, it is not yet clear what role the β polypeptide of the *uncD405* strain plays in restricting proton movement. The residual complex in the *uncD409* mutant, however, completely blocks the proton channel. One aspect of the model for proton-translocating ATPase activity, presented by Kozlov and Skulachev (1977), was the location of the β-subunit deep in the hydrophobic sector during coupled catalysis. The proton channel from the outside was envisaged to interact directly with lysyl groups in the β-subunit (see Fig. I.1c). Such a model would explain the behaviour of an altered β-subunit in preventing proton movement, as well as its inaccessibility to the hypothetical protease.

(f) The effects of polar mutations in the *unc* operon

The polar mutation *unc-436* results in the lack of expression

of the *uncDC* portion of the operon (see Chapter VIII). Correspondingly, it was found that the membranes were not leaky to protons, even after low-ionic strength treatment without PAB. Nor could the membranes bind added Mg-ATPase. The conclusion was drawn that there was either a shortened β fragment produced which blocked the proton channel and the binding site, or that there were gene product(s) from this region which normally contribute to the formation of the proton channel and the binding site.

It is worthwhile, therefore to compare these properties with those of other polar mutants. Strain N_{I44} (Table I.1) appears to be such a mutant. The membranes from this neomycin-resistant strain appear to be somewhat leaky to protons, judging by the extent of NADH-induced acridine fluorescnece quenching (Nieuwenhuis *et al*, 1973). DCCD restores the proton impermeability, but normal Mg-ATPase cannot bind to the membranes (Table I.1). The section of the operon coding for the α-, β- (and γ-) subunits appears to be affected in this strain (see Kanner *et al*, 1975). Thus, strain N_{I44} differs from strain AN1007 (*unc-436*) in at least two respects: firstly, the α-subunit is produced in the latter strain, and secondly, the latter strain is not leaky to protons.

One class of Mu-induced polar mutant strains (see Table I.3), the $uncB^{+}E^{+}A^{+}D^{-}C^{-}$ class, may also be compared with the *unc-436* mutant. Like the other Mu-induced mutant strains, the $uncD^{-}C^{-}$ mutants have membranes which are not leaky to protons, as judged by maximal levels of NADH-induced atebrin fluorescence quenching (Gibson *et al*, 1978). The reconstitution behaviour and the effect of DCCD in these membranes is not yet known; it could well be that some of these strains behave

similarly to the *unc-436* mutant.

Although it is too early yet to draw any firm conclusions from the above comparisons, it is clear that polar mutants will prove useful in characterizing the *unc* operon.

(g) The mixing of Mg-ATPase subunits in partial diploid strains

The fact that genetic complementation occurs between the *uncA401* and *uncD409* alleles (Cox *et al*, 1978a) suggests that there is random mixing of subunits synthesized from either operon. Such mixing would result in a proportion of 1:15 normal Mg-ATPase: Mg-ATPase containing a defective subunit, assuming a 2:2:2:2:2 subunit stoichiometry. If the latter aggregates were inactive, it would account for the small colonies formed by this strain. There is, however, the additional complication of possible non-assembly of some of the defective complexes. The work with the partial diploid strains has shown that defective complexes are assembled to a certain extent, for instance, in strain AN821 (*uncD409/unc*$^{+}$) (Chapter VI) and strain AN785 (*uncA401/uncD405*) (Chapter VII).

It thus appears that, with regard to *uncA/uncD* partial diploid strains, the more effectively the abnormal polypeptides assemble into a complex, the less apparent complementation will occur. Thus, the (*uncA401/uncD405*) and (*uncA401/unc-441(D*$^{-}$*)*) partial diploid strains grow very poorly on succinate, and form small colonies. In the former strain (AN785), the *uncD405* gene product is assembled into Mg-ATPase aggregates (see Chapter VII). In contrast, the (*uncA401/unc-436(D*$^{-}$*C*$^{-}$*)*)

strain grows well on succinate (Fig. VIII.2). If there is any abnormal β produced in the *unc-436* mutant, it is considerably smaller than the normal β-subunit and probably could not participate in the assembly of a Mg-ATPase aggregate.

The speculative ideas discussed in this chapter give some indication of the type of information which can be obtained using the biochemical genetic approach, coupled with protein chemical techniques. Future work should provide further insight into the biosynthesis of the Mg-ATPase, its structure, and the mechanisms of catalysis.

BIBLIOGRAPHY

Abrams, A. (1965) *J. Biol. Chem. 240* , 3675-3681.

Abrams, A., & Smith, J.B. (1974) in *"The Enzymes"* (Boyer, P.D., ed), X, 3rd ed., pp. **395 - 429,** Academic Press Inc. New York and London.

Abrams, A., Jensen, C., & Morris, D.H. (1976) *Biochem. Biophys. Res. Commun. 69*, 804-811.

Adler, J. (1975) *Annu. Rev. Biochem. 44*, 341-356.

Adler, L.W., & Rosen, B.P. (1976) *J. Bacteriol. 128*, 248-256.

Adolfsen, R., & Moudrianakis, E.N. (1976) *Biochemistry 15*, 4163-4170.

Adolfsen, R., McClung, J.A., & Moudrianakis, E.N.(1975) *Biochemistry 14*, 1427-1435.

Ahlers, J., & Günther, T. (1975a) *Z. Naturforsch 30c*, 412-416.

Ahlers, J., & Günther, T. (1975b) *Arch. Biochem. Biophys. 171*, 163-169.

Ahlers, J., Günther, T., & Schrandt, I. (1976) *Z. Naturforsch. 31c*, 201-202.

Altendorf. K. (1977) *FEBS Lett. 73*, 271-275.

Altendorf, K., & Zitzmann, W. (1975) *FEBS Lett. 59*, 268-272.

Altendorf, K., Harold, F.M., & Simoni, R.D. (1974) *J. Biol. Chem. 249*, 4587-4593.

Ames, G.F.-L., & Nikaido, K. (1976) *Biochemistry 15*, 616-623.

Archbold, G.P.R., Farrington, C.L., McKay, A.M., & Malpress, F.H. (1976) *Biochem. Soc. Trans. 4*, 91-94.

Avron, M. (1960) *Biochim. Biophys. Acta 40*, 257-272.

Azocar, O., & Muñoz, E. (1977) *Biochim. Biophys. Acta 482*, 438-452.

Bachmann, B.J., Low, K.B., & Taylor, A.L. (1976) *Bacteriol. Rev. 40*, 116-167.

Bachorik, P.S., Kwiterovich, P.O., & Simon, A (1974) *Anal. Biochem. 60*, 631-636.

Beechey, R.B., & Cattell, K.J. (1973) *Curr. Topics Bioenerg. 5*, 305-357.

Beechey, R.B., Holloway, C.T., Knight, I.G., & Roberton, A.M. (1966) *Biochem. Biophys. Res. Commun. 23*, 75-80.

Beechey, R.B., Hubbard, S.A., Linnett, P.E., Mitchell, A.D., & Munn, E.A. (1975) *Biochem. J. 148*,533-537.

Bell, R.P. (1959) *"The Proton in Chemistry"*, Methuen, London.

Berden, J.A. & Voorn-Brouwer, M.M. (1978) *Biochim. Biophys. Acta 501*, 424-439.

Berger, E.A. (1973) *Proc. Nat. Acad. Sci. USA 70*, 1514-1518.

Bhattacharyya, P., & Barnes, E.M. (1976) *J. Biol. Chem. 251*, 5614-5619.

Bode, H.-J. (1977) *Anal. Biochem. 83*, 364-371.

Boonstra, J., Gutnick, D.L., & Kaback, H.R. (1975) *J. Bacteriol. 124*, 1248-1255.

Boyer, P.D. (1975) *FEBS Lett 58*, 1-6.

Boyer, P.D., Chance, B. Ernster, L., Mitchell, P., Racker, E., & Slater, E.C. (1977) *Annu. Rev. Biochem. 46*, 955-1026.

Bragg, P.D., & Hou, C. (1967) *Can. J. Biochem. 45*,1107-1124.

Bragg, P.D., & Hou, C. (1972) *FEBS Lett, 28*, 309-312.

Bragg, P.D., & Hou, C. (1973) *Biochem. Biophys. Res. Commun. 50*, 729-736.

Bragg, P.D., & Hou, C. (1975) *Arch. Biochem. Biophys. 167*, 311-321.

Bragg, P.D., & Hou, C. (1976a) *Arch. Biochem. Biophys. 174*, 553-561.

Bragg, P.D., & Hou, C. (1976b) *Biochem. Biophys. Res. Commun. 72*, 1042-1048.

Bragg, P.D., & Hou, C. (1977) *Arch. Biochem. Biophys. 178*, 486-494.

Bragg, P.D., Davies, P.L., & Hou, C. (1973) *Arch. Biochem. Biophys. 159*, 664-670.

Brenner, S., Barnett, L., Katz, E.R., & Crick, F.H.C. (1967) *Nature 213*, 449-450.

Bruni, A., Pitotti, A., Contessa, A.R., & Palatini, P. (1971) *Biochem. Biophys. Res. Commun. 44*, 268-274.

Burns, D.D., & Midgley, M. (1976) *Eur. J. Biochem. 67*, 323-333.

Butlin, J.D. (1972) Ph.D. Dissertation, Australian National University.

Butlin, J.D., Cox, G.B., & Gibson, F. (1971) *Biochem. J. 124*, 75-81.

Butlin, J.D., Cox, G.B., & Gibson, F. (1973) *Biochim. Biophys. Acta. 292*, 366-375.

Carriera, J., & Muñoz, E. (1975) *Molecular and Cellular Biochem. 9*, 85-95.

Carriera, J., Leal, J.A., Rojas, M., & Muñoz, E. (1973) *Biochim. Biophys. Acta. 307*, 541-556.

Cattell, K.J., Lindop, C.R., Knight, I.G., & Beechey, R.B. (1971) *Biochem. J. 125*, 169-177.

Chrambach, A., & Rodbard, D. (1971) *Science, 172*, 440-451.

Clarke, D.J., & Morris, J.G. (1976) *Biochem. J. 154*, 725-729.

Contaxis, C.C. & Reithel, F.J. (1971) *J. Biol. Chem. 246*, 677-685.

Cox, G.B., & Downie, J.A. (1978) *Methods in Enzymology* (in press).

Cox, G.B., & Gibson, F. (1974) *Biochim. Biophys. Acta. 346*, 1-25.

Cox, G.B., Newton, N.A., Gibson, F., Snoswell, A.M., & Hamilton, J.A. (1970) *Biochem J. 117*, 551-562.

Cox, G.B., Newton, N.A., Butlin, J.D., & Gibson, F. (1971) *Biochem. J. 125*, 489-493.

Cox, G.B., Gibson, F., McCann, L.M., Butlin, J.D., & Crane, F.L. (1973a) *Biochem. J. 132*, 689-695.

Cox, G.B., Gibson, F., & McCann, L. (1973b) *Biochem J. 134*, 1015-1021.

Cox, G.B., Gibson, F., & McCann, L. (1974) *Biochem. J. 138*, 211-215.

Cox, G.B., Crane, F.L., Downie, J.A., & Radik, J. (1977) *Biochim. Biophys. Acta. 462*, 113-120.

Cox, G.B., Downie, J.A., Gibson, F., & Radik, J. (1978a) *Biochem J. 170*, 593-598.

Cox, G.B., Downie, J.A., Fayle, D.R.H., Gibson, F., & Radik, J. (1978b) *J. Bacteriol. 133*, 287-292.

Dale, G., & Latner, A.L. (1969) *Clin. Chim. Acta. 24*, 61-68.

Daniel, J., Roisin, M.-P., Burstein, C., & Kepes, A. (1975) *Biochim. Biophys. Acta. 376*, 195-209.

Danon, A., & Stoeckenius, W. (1974) *Proc. Nat. Acad. Sci. USA 71*, 1234-1238.

Darnall, D.W., & Klotz, I.M. (1975) *Arch. Biochem. Hiophys. 166*, 651-682.

Davies, P.L., & Bragg, P.D. (1972) *Biochim. Biophys. Acta. 266*, 273-284.

Davis, B.J. (1964) *Ann. N.Y. Acad. Sci. 121*,404-427.

Deamer, D.W., Prince, R.C., & Crofts, A.R. (1972) *Biochim. Biophys. Acta. 274*, 323-335.

Douglas, M.G., Koh, Y., Dockter, M.E., & Schatz, G. (1977) *J. Biol. Chem. 252*, 8333-8335.

Emes, A.V., Latner, A.L., & Martin, J.A. (1975) *Clin. Chim. Acta, 64*, 69-78.

Emes, A.V., Latner, A.L., Martin, J.A., & Mulligan, F.A. (1976) *Biochim. Biophys. Acta, 420*, 57-68.

Enns, R.K., & Criddle, R.S. (1977) *Arch. Biochem. Biophys. 182*, 587-600.

Ernster, L., Carlsson, C., & Boyer, P.D. (1977) *FEBS Lett 84*, 283-286.

Evans, D.J. (1969) *J. Bacteriol. 100*, 914-922.

Evans, D.J. (1970) *J. Bacteriol. 104*, 1203-1212.

Ferguson, S.J. (1977) *Biochem. Soc. Trans, 5*, 582-588.

Ferguson, S.J., Lloyd, W.J., & Radda, G.K. (1975) *Eur. J. Biochem. 54*, 127-133.

Ferguson, S.J., Harris, D.A., & Radda, G.K. (1977) *Biochem J. 162*, 351-357.

Fillingame, R.H. (1975) *J. Bacteriol. 124*, 870-883.

Fillingame, R.H. (1976) *J. Biol Chem. 251*, 6630-6637.

Fiolet, J.W.T., Bakker, E.P., & Van Dam, K. (1974) *Biochim. Biophys. Acta. 368*, 432-445.

Freese, E., Bautz-Freese, E., & Bautz, E. (1961) *J. Mol. Biol. 3*, 133-143.

Friedl, P., Schmid, B.I., & Schairer, H.U. (1976) *Eur. J. Biochem. 73*, 461-468.

Futai, M., Sternweis, P.C., & Heppel, L.A. (1974) *Proc. Nat. Acad. Sci. USA 71*, 2725-2729.

Garen, A. (1968) *Science 160*, 149-150.

Garland, P.B. (1977) in *"Microbial Energetics"*, (Haddock, B.A., & Hamilton W.A., eds), pp.1-21. Cambridge University Press, Cambridge.

Garland, P.B., & Haddock, B.A. (1977) *Biochem. Soc. Trans. 5*, 479-484.

Gibson, F. & Cox, G.B. (1973) *Essays in Biochemistry 9*, 1-29.

Gibson, F., Cox, G.B., Downie, J.A., & Radik, J. (1977a) *Biochem J. 162*, 665-670.

Gibson, F., Cox, G. B., Downie, J.A., & Radik, J. (1977b) *Biochem. J. 164*, 193-198.

Gibson, F., Downie, J.A., Cox, G.B., & Radik, J. (1978) *J. Bacteriol. (in press)*.

Giordano, G., Riviere, C., & Azoulay, E. (1975) *Biochim Biophys. Acta. 389*, 203-218.

Goffeau, A. Landry, Y., Foury, F., Briquet, M., & Colson, A.-M. (1973) *J. Biol. Chem. 248*, 7097-7105.

Gomori, G. (1939) *Proc. Exper. Biol. Med. 42*, 23-26.

Gould, J.M., & Cramer, W.A. (1977) *J. Biol. Chem. 252*, 5875-5882.

Griffiths, D.E. (1976) *Biochem. J. 160*, 809-812.

Griffiths, D.E., & Hyams, R.L. (1977) *Biochem. Soc. Trans. 5*, 207-208.

Griffiths, D.E., Cain, K., & Hyams, R.L. (1977a) *Biochem. Soc. Trans. 5*, 205-207.

Griffiths, D.E., Hyams, R.L., & Bertoli, E. (1977b) *FEBS Lett.74*, 38-42.

Griffiths, D.E., Hyams, R.L., Bertoli, E., & Carver, M. (1977c) *Biochem. Biophys. Res. Commun. 75*, 449-456.

Griffiths, D.E., Hyams, R.L. & Partis, M.D. (1977d) *FEBS Lett. 78*, 155-160.

Grinius, L., Slusnyte, R., & Griniuviene, B. (1975) *FEBS Lett. 57*, 290-293.

Günther, Th., & Mariss, G. (1974) *Z. Naturforsch 29c*, 60-62.

Günther, Th., Pellnitz, W., & Mariss, G. (1974) *Z. Naturforsch, 29c*, 54-59.

Gutnick, D.L., Kanner, B.I., & Postma, P.W. (1972) *Biochim. Biophys. Acta. 283*, 217-222.

Haddock, B.A., & Begg, Y.A. (1977) *Biochem. Biophys, Res. Commun. 79*, 1150-1154.

Haddock, B.A., & Downie, J.A. (1974) *Biochem. J. 142*, 703-706.

Haddock, B.A., & Jones, C.W. (1977) *Bacteriol. Rev. 41*, 47-99.

Hamilton,W.A. (1977) in *"Microbial Energetics"*, (Haddock, B.A., & Hamilton, W.A., eds), pp. 185-216, Cambridge University Press, Cambridge.

Hanson, R.L., & Kennedy, E.P. (1973) *J. Bacteriol. 114*, 772-781.

Hanstein, W.G., & Hatefi, Y. (1974) *J. Biol. Chem. 249*, 1356-1362.

Hare, J.F. (1975) *Biochem. Biophys. Res. Commun. 66*, 1329-1337.

Harold, F.M. (1972) *Bacteriol. Rev. 36*, 172-230.

Harold, F.M. (1977a) *Curr. Topics Bioenerg. 6*, 83-149.

Harold, F.M. (1977b) *Annu. Rev. Microbiol. 31*, 181-203.

Harold, F.M., Baarda, J.R., Baron, C., & Abrams, A. (1969) *J. Biol. Chem. 244*, 2261-2268.

Harris, D.A. (1978) *Biochim. Biophys. Acta. 463*,245-273.

Hasan, S.M., & Rosen, B.P. (1977) *Biochim. Biophys. Acta. 459*, 225-240.

Hasan, S.M., Tsuchiya, T., & Rosen, B.P. (1978) *J. Bacteriol. 133*, 108-113.

Hatefi, Y. (1975) *J. Supramol. Struct. 3*, 201-213.

Hayes, W. (1968) *"The Genetics of Bacteria and their Viruses"* Blackwell Scientific Publications, Oxford and Edinburgh.

Hempfling, W.P. (1970) *Biochim. Biophys. Acta. 205*, 169-182.

Hertzberg, E.L., & Hinkle, P.C. (1974) *Biochem. Biophys. Res. Commun. 58*, 178-184.

Higashi, T., Kalra, V.K., Lee, S.H., Bogin, E., & Brodie, A.F. (1975) *J. Biol. Chem. 250*, 6541-6548.

Holzer, H., Betz, H., & Ebner, E. (1975) *Current Topics in Cellular Regulation, 9*, 103-156.

Hong, J. -S. (1977) *J. Biol. Chem. 252*, 8582-8588.

Hong, J. -S., & Ames, B.N. (1971) *Proc. Nat. Acad. Sci. USA. 68*, 3158-3162.

Hong, J. -S., & Kaback, H.R. (1972) *Proc. Nat. Acad. Sci. USA. 69*, 3336-3340.

Howe, M.M., & Bade, E.G. (1975), *Science, 190*, 624-632.

Hulla, F.W., Höckel, M., Risi, S., & Dose, K.(1976) *Eur. J. Biochem. 67*, 469-476.

Jagendorf, A.T., & Uribe, E. (1966) *Proc. Nat. Acad. Sci. USA. 55*, 170-177.

Jones, C.W. (1977) in *"Microbial Energetics"* (Haddock, B.A. & Hamilton, W.A., eds), pp. 23-59. Cambridge University Press, Cambridge.

Jones, C.W., Brice, J.M., & Edwards, C. (1977) *Arch, Microbiol. 115*, 85-93.

Kagawa, Y., Sone, N., Yoshida, M., Hirata, H., & Okamoto, H. (1976) *J. Biochem. 80*, 141-151.

Kalra, V.K., & Brodie, A.F. (1971) *Arch. Biochem. Biophys. 147*, 653-659.

Kanner, B.I., & Gutnick, D.L. (1972a) *J. Bacteriol. 111*, 287-289.

Kanner, B.I., & Gutnick, D.L. (1972b) *FEBS Lett. 22*, 197-199.

Kanner, B.I., Nelson, N., & Gutnick, D.L. (1975) *Biochim. Biophys. Acta. 396*, 347-359.

Kayalar, C., Rosing, J., & Boyer, P.D., (1977) *J. Biol. Chem. 252*, 2486-2491.

Kenrick, K.G., & Margolis, J. (1970) *Anal. Biochem. 33*, 204-207.

King, E.J. (1932) *Biochem. J. 26*, 292-297.

Knowles, A.F., & Penefsky, H.S. (1972a) *J. Biol. Chem. 247*, 6617-6623.

Knowles, A.F., & Penefsky, H.S. (1972b) *J. Biol. Chem. 247*, 6624-6630.

Kobayashi, H. & Anraku, Y. (1972) *J. Biochem. 71*, 387-399.

Kobayashi, H. & Anraku, Y. (1974) *J. Biochem. 76*, 1175-1182.

Kobayashi, H., Kin, E., & Anraku, Y. (1974) *J. Biochem. 76*, 251-261.

Konings, W.N., & Boonstra, J. (1977) *Current Topics in Membranes and Transport 9*, 177-231.

Kort, E.N., Goy, M.F., Larsen, S.H., & Adler, J. (1975) *Proc. Nat. Acad. Sci. USA. 72*, 3939-3943.

Koshland, D.E.(Jr.), (1977) in *"Microbial Energetics"* (Haddock, B.A., & Hamilton W.A., eds), pp.317-331. Cambridge University Press, Cambridge.

Kozlov, I.A., & Mikelsaar, H.N. (1974) *FEBS Lett. 43*, 212-214.

Kozlov, I.A., & Skulachev, V.P. (1977) *Biochim. Biophys. Acta. 463*, 29-89.

Kraayenhof, R. (1970) *FEBS Lett. 6*, 161-165.

Kraayenhof, R., & Fiolet, J.W.T. (1974) in *"Dynamics of Energy-Transducing Membranes"* (Ernster, L., Estabrook, R.W., & Slater, E.C., eds), pp.355- 64, Elsevier, Amsterdam.

Kurzer, F., & Douraghi-Zadeh, K. (1967) *Chem.Revs. 67*, 107-152

Larsen, S.H., Adler, J., Gargus, I.I., & Hogg, R.W. (1974) *Proc. Nat. Acad. Sci. USA. 71*, 1239-1243.

Larson, R.J., & Smith, J.B. (1977) *Biochemistry 16*, 4266-4270.

Lauquin, G., Brandolin, G., & Vignais, P. (1976) *FEBS Lett. 67*, 306-311.

Lee, S.-H., Cohen, N.S., & Brodie, A.F. (1976) *Proc. Nat. Acad. Sci. USA, 73*, 3050-3053.

Lieberman, M.A., & Hong, J.-S. (1974) *Proc. Nat. Acad. Sci. USA 71*, 4395-4399.

Lieberman, M.A., Simon, M., & Hong, J.-S. (1977) *J. Biol. Chem. 252*, 4056-4067.

Linnane, A.W., Groot Obbink, D.J., Kellerman, G.M., Trembath, M.K., & Monk, B.C. (1976) *Proc. Aust. Biochem. Soc. 9*, Q7-Q8.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., & Randall, R.J. (1951) *J. Biol. Chem. 193*, 265-275.

Luria, S.E., & Burrous, J.W. (1957) *J. Bacteriol. 74*, 461-476.

Macko, V., & Stegemann, H. (1969) *Hoppe-Seyler's Z. Physiol. Chem. 350*, 917-919.

Maeda, M., Kobayashi, H., Futai, M., & Anraku, Y. (1976) *Biochem. Biophys. Res. Commun. 70*, 228-234.

Maeda, M., Futai, M., & Anraku, Y. (1977a) *Biochem. Biophys. Res. Commun. 76*, 331-338.

Maeda, M., Kobayashi, H., Futai, M., & Anraku, Y. (1977b) *J. Biochem. 82*, 311-314.

Maloney, P.C. (1977) *J. Bacteriol. 132*, 564-575.

Maloney, P.C., Kashket, E.R., & Wilson, T.H. (1974) *Proc. Nat. Acad. Sci. USA 71*, 3896-3900.

Marahiel, M.A., Imam, G., Nelson, P., Pieniazek, N.J., Stepien, P.P., & Küntzel, H. (1977) *Eur. J. Biochem. 76*, 345-354.

Mares-Guia, M., & Shaw. E. (1965) *J. Biol. Chem. 240*, 1579-1584.

McCarty, R.E., & Racker, E. (1967) *J. Biol. Chem. 242*, 3435-3439.

Miller, D.W., & Elgin, S.C.R. (1974) *Anal. Biochem. 60*, 142-148.

Miller, K.R., & Staehelin, L.A. (1976) *J. Cell Biol. 68*, 30-47.

Mitchell, P. (1966) *Biol. Revs. 41*, 445-502.

Mitchell, P. (1976) *Biochem. Soc. Trans. 4*, 399-430.

Mitchell, P. (1977) *FEBS Lett. 78*, 1-20.

Monod, J., Cohen-Bazire, G., & Cohn, M. (1951) *Biochim. Biophys. Acta. 7*, 585-599.

Muller, J.L.M., Rosing, J., & Slater, E.C. (1977) *Biochim. Biophys. Acta. 462*, 422-437.

Murgola, E.J., & Yanofsky, C. (1974) *J. Bacteriol. 117*, 444-448.

Nagle, J.F., & Morowitz, H.J. (1978) *Proc. Nat. Acad. Sci. USA, 75*, 298-302.

Nelson, N. (1976) *Biochim. Biophys. Acta. 456*, 314-338.

Nelson, N., Nelson, H., & Racker, E. (1972) *J. Biol. Chem. 247*, 7657-7662.

Nelson, N., Kanner, B.I., & Gutnick, D.L. (1974) *Proc. Nat. Acad. Sci. USA 71*, 2720-2724.

Nieuwenhuis, F.J.R.M., & Bakkenist, A.R.J. (1977) *Biochim. Biophys. Acta. 459*, 596-604.

Nieuwenhuis, F.J.R.M., Kanner, B.I., Gutnick, D.L., Postma, P.W., & Van Dam, K. (1973) *Biochim. Biophys. Acta 325*, 62-71.

Nieuwenhuis, F.J.R.M., Thomas, A.A.M., & Van Dam, K. (1974a) *Biochem. Soc. Trans. 2*, 512-513.

Nieuwenhuis, F.J.R.M., Drift, J.A.M.v.d., Voet, A.B., & Van Dam, K. (1974b) *Biochim. Biophys. Acta. 368*, 461-463.

Novick, R.P., Clowes, R.D., Cohen, S.N., Curtiss, R., Datta, N. & Falkow, S. (1976) *Bacteriol. Rev. 40*, 168-189.

Oesterhelt, D., Gottschlich, R., Hartmann, R., Michel, M., & Wagner, G. (1977) in *"Microbial Energetics"* (Haddock, B.A. & Hamilton, W.A., eds), pp.333-349, Cambridge University Press, Cambridge.

O'Farrell, P.H. (1975) *J. Biol. Chem. 250*, 4007-4021.

Ohta, S., Nakanishi, M., Tsuboi, M., Yoshida, M., & Kagawa, Y. (1978) *Biochem. Biophys. Res. Commun. 80*,929-935.

Okamoto, H.,Sone, N., Hirata, H., Yoshida, M., & Kagawa. Y. (1977) *J. Biol. Chem. 252*, 6125-6131.

Or, A., Kanner, B.I., & Gutnick, D.L. (1973) *FEBS Lett. 35*, 217-219.

Ornstein, L. (1964) *Ann. N.Y. Acad. Sci. 121*, 321-349.

Ort, D.R., & Dilley, R.A. (1976) *Biochim. Biophys. Acta. 449*, 95-107.

Ort, D.R., Dilley, R.A., & Good, N.E. (1976) *Biochim. Biophys. Acta. 449*, 108-124.

Overath, P., & Träuble, H. (1973) *Biochemistry 12*, 2625-2634.

Panet, R., & Sanadi, D.R. (1976) *Current Topics in Membranes and Transport, 8*, 99-150.

Partis, M.D., Bertoli, E., Mascarello, S., & Griffiths, D.E. (1976) *Biochem. Soc. Trans. 4*, 88-91.

Partis, M.D., Hyams, R.L., & Griffiths, D.E. (1977) *FEBS Lett. 75*, 47-51.

Patel, L., & Kaback, H.R. (1976) *Biochemistry 15*, 2741-2746.

Patel, L., Schuldiner, S., & Kaback, H.R. (1975) *Proc. Nat. Acad. Sci. USA, 72*, 3387-3391.

Pedersen, P.L. (1975) *(J.) Bioenergetics 6*, 243-275.

Peter, H.W., & Ahlers, J. (1975) *Arch. Biochem. Biophys. 170*,169-178.

Pinchot, G.B. (1953) *J. Biol. Chem. 205*, 65-74.

Pittard, J. (1965) *J. Bacteriol. 89*, 680-686.

Porat, N., Ben-Shaul, Y., & Friedberg, I. (1976) *Biochim. Biophys. Acta, 440*, 365-376.

Prezioso, G., Hong, J.-S., Kerwar, G.K., & Kaback, H.R. (1973) *Arch. Biochem. Biophys. 154*, 575-582.

Pringle, J.R. (1975) *Methods in Cell Biology 12*, 149-184.

Pullman, M.E., & Monroy, G.C. (1963) *J. Biol. Chem. 238*, 3762-3769.

Racker, E. (1967) *Fed. Proc. 26*, 1335-

Ramos, S., Schuldiner, S., & Kaback, H.R. (1976) *Proc. Nat. Acad, Sci., USA 73*, 1892-1896.

Regnier, P., & Thang, M.N. (1973) *FEBS Lett. 36*, 31-33.

Riccio, P., Aquila, H., & Klingenberg, M. (1975) *FEBS Lett. 56*, 133-138.

Righetti, P.G., & Caravaggio, T. (1976) *J. Chromatog. 127*, 1-28.

Righetti, P.G., & Drysdale, J.W. (1976) in *"Laboratory Techniques in Biochemistry and Molecular Biology"* (Work, T.S., & Work, E., eds), Vol. 5., pp. 335-585, North-Holland, Amsterdam.

Risi, S., Höckel, M., Hulla, F.W., & Dose, K. (1977) *Eur. J. Biochem. 81*, 103-109.

Roberton, A.M., Holloway, C.T., Knight, I.G. & Beechey, R.B. (1968) *Biochem J. 108*, 445-456.

Robertson, R.N. (1978) in (title tentative) *"Light-trapping Membranes"* (in press), Academic Press, N.Y. and London.

Robertson, R.N., & Boardman, N.K. (1975) *FEBS Lett. 60*, 1-6.

Robertson, R.N., & Thompson, T.E. (1977) *FEBS Lett. 76*, 16-19.

Rodbard, D., Kapadia, G., & Chrambach, A. (1971) *Anal. Biochem. 40*, 135-157.

Roisin, M.-P., & Kepes, A. (1972) *Biochim. Biophys. Acta. 275*, 333-346.

Roisin, M.-P., & Kepes, A. (1973) *Biochim. Biophys. Acta. 305*, 249-259.

Rosen, B.P. (1973a) *J. Bacteriol. 116*, 1124-1129.

Rosen. B.P. (1973b) *Biochem. Biophys. Res. Commun. 53*, 1289-1296.

Rosen, B.P., & Adler, L.W. (1975) *Biochim. Biophys. Acta. 387*, 23-36.

Rosenberg, H., Cox, G.B., Butlin, J.D., & Gutowski, S.J. (1975) *Biochem J. 146*, 417-423.

Rosing, J., Kayalar, C., & Boyer, P.D. (1977) *J. Biol. Chem. 252*, 2478-2485.

Russo, J.A., Lamos, C.M., & Mitchell, R.A. (1978) *Biochemistry 17*, 473-480.

Ryrie, I.J. (1975a) *Arch. Biochem. Biophys. 168*, 704-711.

Ryrie, I.J. (1975b) *Arch. Biochem. Biophys. 168*, 712-714.

Ryrie, I.J. (1977) *Arch. Biochem. Biophys. 184*, 464-475.

Ryrie, I.J., & Blackmore, P.F. (1976) *Arch. Biochem. Biophys. 176*, 127-135.

Ryrie, I.J., & Jagendorf, A.T. (1972) *J. Biol. Chem. 247*, 4453-4459.

Schairer, H.U., & Gruber, D. (1973) *Eur. J. Biochem. 37*, 282-286.

Schairer, H.U., & Haddock, B.A. (1972) *Biochem. Biophys. Res. Commun. 48*, 544-551.

Schairer, H.U., Friedl, P., Schmid, B.I., & Vogel, G. (1976) *Eur. J. Biochem, 66*, 257-268.

Sebald, W. (1977) *Biochim. Biophys. Acta. 463*, 1-27.

Senior, A.E. (1973) *Biochim. Biophys. Acta. 301*, 249-277.

Senior, A.E. (1978) in *"Membrane Proteins in Energy Transduction"* (Capaldi, R.A., ed), Marcel Dekker Inc., New York (in press).

Senior, A.E., & MacLennan, D.H. (1970) *J. Biol. Chem. 245*, 5086-5095.

Serrano, R., Kanner, B.I., & Racker, E. (1976) *J. Biol. Chem. 251*, 2453-2461.

Sierra, M.F., & Tzagoloff, A. (1973) *Proc. Nat. Acad. Sci. USA 70*, 3155-3159.

Simoni, R.D., & Postma, P.W. (1975) *Annu. Rev. Biochem. 44*, 523-554.

Simoni, R.D., & Shallenberger, M.K. (1972) *Proc. Nat. Acad. Sci. USA 69*, 2663-2667.

Simoni, R.D., & Shandell, A. (1975) *J. Biol. Chem. 250*, 9421-9427.

Smith, J.B., & Sternweis, P.C. (1975) *Biochem. Biophys. Res. Commun. 62*, 764-771.

Smith, J.B. & Sternweis, P.C. (1977) *Biochemistry 16*, 306-311.

Smith, J.B., Sternweis, P.C., & Heppel, L.A. (1975) *J. Supramol, Struct. 3*, 248-255.

Snoswell, A.M. (1966) *Biochemistry 5*, 1660-1666.

Sone, N., Yoshida, M., Hirata, H., & Kagawa, Y. (1975) *J. Biol. Chem. 250*, 7917-7923.

Sone, N., Yoshida, M., Hirata, H., Okamoto, H., & Kagawa, Y. (1976) *J. Membrane. Biol. 30*, 121-134.

Sone, N., Yoshida, M., Hirata, H., & Kagawa, Y. (1977a) *J. Biochem. 81*, 519-528.

Sone, N., Yoshida, M., Hirata, H., & Kagawa, Y. (1977b) *J. Biol. Chem. 252*, 2956-2960.

Springer, M.S., Kort, E.N., Larsen, S.H., Ordal, G.W., Reader, R.W., & Adler, J. (1975) *Proc. Nat. Acad. Sci.USA 72*, 4640-4644.

Stekhoven, F.S., Waitkus, R.F., & Van Moerkerk, H. Th. B. (1972) *Biochemistry 11*, 1144-1150.

Sternweis, P.C., & Smith J.B. (1977) *Biochemistry 16*, 4020-4025.

Stiggall, D.L., Galante, Y., & Hatefi, Y. (1978) *J. Biol. Chem. 253*, 956-964.

Stouthamer, A.H. (1977) in *"Microbial Energetics"* (Haddock, B.A., & Hamilton, W.A., eds), pp.285-315, Cambridge University Press, Cambridge.

Sun, I.L., Phelps, D.C., & Crane, F.L. (1975) *FEBS Lett. 54*, 253-258.

Takayama, K., MacLennan, D.H., Tzagoloff, A., & Stoner, C.D. (1964) *Arch. Biochem. Biophys. 114*, 223-230.

Takeshige, K., Hess, B., Böhm, M., and Zimmermann-Telschow, H. (1976) *Hoppe-Seyler's Z. Physiol Chem. 357*, 1605-1622.

Taylor, A. L. (1963) *Proc. Nat. Acad. Sci. USA 50*, 1043-1051.

Taylor A.L., & Thoman, M. S., (1964) *Genetics 50*, 659-677.

Thipayathasana, P. (1975) *Biochim. Biophys. Acta. 408*, 47-57.

Thipayathasana, P., & Valentine, R.C. (1974) *Biochim. Biophys. Acta. 347*, 464-468.

Tomochika, K. -I., & Hong, J. -S. (1978) *J. Bacteriol. 133*, 1008-1014.

Träuble, H., & Eibl, H. (1974) *Proc. Nat. Acad. Sci. USA 71*, 214-219.

Träuble, H., & Overath, P. (1973) *Biochim. Biophys. Acta. 307*, 491-512.

Tsuchiya, T. (1977) *J. Bacteriol. 129*, 763-769.

Tsuchiya, T., & Rosen, B.P. (1975a) *Biochem. Biophys. Res. Commun. 63*, 832-838.

Tsuchiya, T., & Rosen, B.P. (1975b) *J. Biol. Chem. 250*, 8409-8415.

Tsuchiya, T., & Rosen, B.P. (1976) *Biochem. Biophys. Res. Commun. 68*, 497-502.

Tzagoloff, A. (1971) *Current Topics in Membranes and Transport 2*, 157-205.

Tzagoloff, A., & Akai, A. (1972) *J. Biol. Chem. 247*, 6517-6523.

Tzagoloff, A., & Meagher, P. (1971) *J. Biol. Chem. 246*, 7328-7336.

Tzagoloff, A., Rubin, M.S., & Sierra, M.F. (1973) *Biochim. Biophys. Acta. 301*, 71-104.

Tzagoloff, A., Akai, A., & Foury, F. (1976) *FEBS Lett. 65*, 391-395.

Uribe, E.G. (1972) *Biochemistry 11*, 4228-4235.

Van de Stadt, R.J., De Boer, B.L., & Van Dam, K. (1973) *Biochim. Biophys. Acta. 292*, 338-349.

Van Thienen, G., & Postma, P.W. (1973) *Biochim. Biophys. Acta.323*, 429-440.

Verschoor, G.J., Van Der Sluis, P.R., & Slater, E.C.(1977) *Biochim. Biophys. Acta. 462*, 438-449.

Vignais, P.V., Vignais, P.M., & Doussiere, J. (1975) *Biochim. Biophys. Acta. 376*, 219-230.

Vogel, G. & Steinhart, R. (1976) *Biochemistry 15*, 208-216.

Wachstein, M., & Meisel, E. (1957) *Amer. J. Clin. Path. 27*,13-23.

Weinbaum, G., & Markman, R. (1966) *Biochim. Biophys. Acta. 124*, 207-209.

West, I.C., & Mitchell, P. (1974) *FEBS Lett. 40*, 1-4.

Williams, R.J.P. (1978) *FEBS Lett. 85*, 9-19.

Wilson, D.M., Alderete, J.F., Maloney, P.C. & Wilson, T.H. (1976) *J. Bacteriol. 126*, 327-330.

Wilson, G., & Fox, C.F. (1971) *J. Mol. Biol. 55*, 49-60.

Witt, H.T., Schlodder, E., & Gräber, P. (1976) *FEBS Lett. 69*, 272-276.

Wong, S.C.K., Hall, D.C., & Josse, J. (1970) *J. Biol. Chem. 245*, 4335-4345.

Wright, G.L., Farrell, K.B., & Roberts, D.B. (1973) *Biochim. Biophys. Acta. 295*, 396-411.

Yamamoto, T.H., Mevel-Ninio, M., & Valentine, R.C. (1973) *Biochim. Biophys. Acta. 314*, 267-275.

Yoshida, M., Sone, N., Hirata, H., Kagawa, Y., Takeuchi, Y., & Ohno, K. (1975) *Biochem. Biophys. Res. Commun. 67*, 1295-1300.

Yoshida, M., Sone, N., Hirata, H., & Kagawa, Y. (1977a) *J. Biol. Chem.252*, 3480-3485.

Yoshida, M., Okamoto, H., Sone, N., Hirata, H., & Kagawa, Y. (1977b) *Proc. Nat. Acad. Sci. USA 74*, 936-940.

Zukin, R.S., & Koshland, D.E. (1976) *Science 193*, 405-408.